Listing of Claims (marked-up version):

Claim 1 (currently amended): A method for decreasing cholesterol, low-density lipoprotein and triglyceride levels in blood under cholesterol-enriched and normal diet, which comprises administering to a mammal 24-epibrassinolide ~~(EB1), a plant hormone of structural formula I belong to brassinosteroid series~~ in a daily dose of 0.03-200 micrograms per kilogram of body weight.

Claims 2-3 (canceled)

Claim 4 (previously presented): The method of claim 1comprising administering of 24-epibrassinolide during 4-12 weeks.

Claims 5-9 (canceled)

Brassinosteroids:

A New Class of Plant Hormones

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CHAPTER IX

PHYSIOLOGICAL MODE OF ACTION OF BS

Specific physiological action in plants has triggered the interest and curiosity of many researchers to new substances which later became known as BS. Although this subject has been widely discussed in many reviews (Gamburg, 1986; Meudt and Nes, 1987; Mandava, 1988; Marquardt and Adam, 1991; Takeuchi, 1992; Sakurai and Fujioka, 1993; Baiguz and Czerpak, 1995; Prusakova and Chizhova, 1996; Brosa, 1997; Sasse, 1991b, 1996, 1997) and in a previous monograph (Khripach *et al.*, 1993a), new developments in this field are proceeding quickly and regularly need reconsideration. This and the following chapters are a new attempt to analyze the situation in the area at this time taking into account all the data published in the literature to date. Special attention is

paid to the data obtained by investigators from Eastern Europe, which have never been reviewed before and which are accessible with difficulty for international readership.

Nowadays the wide spectrum of biological activity of BS and the complex character of their mode of action are well documented. These features are the result of a cascade of biochemical reactions which can be initiated *via* direct action of BS on the genome or by an extragenetic route. Both routes assume the participation of a system of secondary messengers and can act together. This complexity and interactions of the bioeffects of BS make a classification of these effects rather difficult. The current level of knowledge in this area does not yet allow a systematic approach for the presentation of data based on the mechanism of action of BS, and a combined variant is used. For easier reading the whole plant effects and the effects on physiological processes will be discussed separately. For example, plant growth initiated by BS is discussed separately from the effects on processes such as cell wall loosening, enzyme activation, and protein biosynthesis. A mixed scheme is used for the explanation of phenomena when effects of different levels are involved.

A. GROWTH AND DEVELOPMENT

The best known and the most widely studied biological effect of BS is their ability to stimulate plant growth in a variety of systems such as whole plants, excised segments, cuttings, and seedlings. Research on the physiological mode of action of BS in plants had already begun prior to the structural elucidation of Bl, the first representative of this group of phytohormones (Mitchell *et al.*, 1970; Mitchell and Gregory, 1972; Worley and Mitchell, 1971; Worley and Krizek, 1972; Milborrow and Pryce, 1973; Mandava *et al.*, 1978). The first data were obtained from investigations of the brassin complex. It was shown that when brassins were applied in a concentration of 10 μg per plant, they induced

remarkable elongation of the second and third internodes of intact bean plants. For example, the second internodes grew at an average of 155 mm four days after treatment, while controls grew only 12 mm (Mitchell *et al.*, 1970). Brassins typically caused elongation of all parts of the bean plants, and increased the length of stem, shoots, roots, weight of pods, and quantity of buds (Worley and Krizek, 1972; Mitchell and Gregory, 1972; Gregory, 1981). A study of the effect of brassins on the retardation of the bean hypocotyl hook opening and the reversal of the light inhibition of hypocotyl elongation demonstrated that they exhibit both "auxin"- and "gibberellin"-like activities (Yopp *et al.*, 1979). The results of the research till 1979 have however some restrictions, because a complex mixture of compounds was used. The situation changed after pure Bl was isolated, its structure determined, and its synthesis carried out.

For the investigation of the physiological mode of action of BS many test systems were used that were developed earlier for other phytohormones. It was shown that BS have a wide spectrum of biological action and are capable of influencing various physiological processes in plants. Thus, the observed growth acceleration of bean internodes after treatment with brassins was the result of stimulation of cell elongation and cell division. Later, similar responses, related to cell growth and accumulation of biomass, were found to be typical for BS and were observed in various plants. It was found that Bl induced elongation of pea epicotyls, apical segments of dwarf pea (Sasse, 1988, 1990), mung bean epicotyls (Kamuro and Inada, 1987), segments of azuki bean epicotyls (Mandava *et al.*, 1981), and hypocotyls of cucumber (Katsumi, 1985), sunflower (Mandava *et al.*, 1981), and radish (Choi, C. *et al.*, 1986). Also stimulation of wheat leaves and root growth and growth of mustard seedlings were observed (Braun and Wild, 1984a). These data correlate well with those (Gregory, 1981) obtained for barley plants cultivated from seeds treated with BS. They had larger leaves and stems and grew faster in comparison with control plants. Similar results were obtained in rice (Zhou, A.-Q., 1987; Hirai and Fujii, 1985), barley, lettuce

(Meudt *et al.*, 1983), and celery (Wang, Y. *et al.*, 1988) plants when sprayed with a BS solution. Growth acceleration after BS treatment was observed also for some woody species (Worley and Krizek, 1972) and fungi (Gartz *et al.*, 1990). Experiments with mung bean seedlings (Zhao and Wu, 1990) revealed that stimulation of stem growth after treatment with EBl was mainly the result of cell expansion and connected with enhanced water absorption.

Among all growth responses to BS, the influence on the root system is the least studied and unequivocal due to its variability in different conditions. In contrast to the well-documented growth-promoting effects on aerial tissues, BS caused either growth inhibition or growth promotion in roots. This depends on the part of the plant to which BS are applied, the mode of treatment, the dose, the time of exposure, and so on. In early experiments with mung bean hypocotyls (Morishita *et al.*, 1983) an inhibition of adventitious root growth under the influence of BS was shown, whereas other authors (Romani *et al.*, 1983; Cerana *et al.*, 1985), using cuttings of maize roots, reported growth promotion. A stimulating effect on adventitious root development in terms of number and length was obtained with a very low concentration of EBl when applied to soybean hypocotyl segments (Sathiyamoorthy and Nakamura, 1990).

An opposite effect was obtained in epicotyl and hypocotyl cuttings of mung bean (Guan and Roddick, 1988a). The number and mean length of adventitious roots were decreased after treatment with EBl and significant reduction (approximately 50%) in the total root length was observed with 0.01 μM BS. A similar effect was obtained with tomato cuttings (Guan and Roddick, 1988b). In cultured tomato roots EBl (Roddick *et al.*, 1993) and three other BS (Bl, (22*S*,23*S*)-EBl, and HBl) with different side chains caused an inhibitory effect in concentration range between 10^{-6} and 10^{-10} M. Bl was the most active and evoked inhibition at 10^{-10} M, compared to HBl at 10^{-6} M (Roddick, 1994). The highest response was observed in the main axis. This differed from the situation with intact seedlings of wheat, mung bean, and maize, where inhibition first was

seen on the lateral root development (Roddick and Ikekawa, 1992). In excised rice roots, the action of Bl was shown to depend on the mode of treatment; when supplied to the scutellum, it stimulated growth, whereas application to the root tip caused an inhibitory effect (Radi and Maeda, 1988).

An interesting result was obtained with respect to root formation in cuttings from the *Norway spruce* tree after treatment with synthetic (22*S*,23*S*)-HBl (Rönsch *et al.*, 1993). Cuttings were treated with 60 ppm of BS analog after being harvested in March, stored in darkness, and planted in May. In the controls 50% of the cuttings rooted; in the treated cuttings this number increased to 92%. A promoting effect was observed when rice seeds were soaked in a solution of 10^{-5} and 10^{-3} ppm BS before germination. Root weight and rooting ability were significantly increased also when rice plants at tillering were fed with BS through the roots or by foliar spray (Wang, S.-G., and Deng, 1992). Growth promotion of root explants of young tobacco seedlings was observed when cultured in a medium with 0.01 or 0.05 ppm EBl (Chen *et al.*, 1990). BS were found to promote the growth and rooting of potato cuttings when added to the nutritient solution. HBl, Bl, and EBl (0.02-0.2 mg/l) caused a twofold and higher increase of the number of adventitious roots (Bobrik, 1995).

The variable influence of BS on root development was discussed in comprehensive reviews by Roddick and Guan (1991) and Sasse (1994). Analyzing the responses of different root systems to BS, some common peculiarities can be indicated. For cultured excised roots mostly growth reduction is observed, whereas in cuttings and seedlings where shoot tissues are present, promotion takes place more often, especially when treatment is made *via* aerial tissue. Although the physiological background of root responses to BS is still not clear, investigations in this field are of great value for understanding the role and mechanism of action of both endogenous and exogenous BS in plants. The observation of root growth promoting ability of BS in intact seedlings and plants is important for potential practical application.

In this context special attention should be paid to reports about the enhancement of resistance to stress conditions of plants treated by BS. An example is the increase of root activity, plant growth, and weight of roots and shoots of gram plants (*Cicer arietinum*) under water stress (Singh *et al.*, 1993) after application of 0.001 ppm EBl as a foliar spray. The effects of salt stress can be decreased by soaking seeds in a BS solution before germination. An enhancement of the length and number of roots of rice plants cultivated under salt stress conditions from seeds soaked in a HBl solution was reported (Uesono *et al.*, 1985; Takematsu and Takeuchi, 1989).

Along with growth alteration, BS usually influence other aspects of plant development. In particular, their effect on reproduction, maturation, senescence, and seed yield has been noticed. For example, Bl induced the formation of bisexual and pistillate flowers in *Luffa cylindrica* staminate inflorescences and slightly promoted flowering in nonvernalized, but not vernalized, radish plants (Suge, 1986). EBl strongly retarded maturation and senescence in hypocotyl segments of mung bean seedlings (Zhao *et al.*, 1987), and it efficiently enhanced cell reproduction and colony formation of Chinese cabbage mesophyll protoplasts (Nakajima *et al.*, 1996). The biosynthesis of BS was suggested to be essential for the differentiation of cells into tracheary elements in isolated *Zinnia* mesophyll cells (Iwasaki and Shibaoka, 1991) and for inducing entry into the final stage of differentiation (Yamamoto *et al.*, 1997). Recently, an exclusive role of BS in plant development was demonstrated in mutants defective in Bl synthesis or response to it, which showed dramatically changed phenotypes (Clouse, 1996a, 1997). The increase of BS content in plant pollen with maturity also confirms this role (Asakawa *et al.*, 1996).

The data on the influence of BS on seed yields are quite extensive and will be discussed in detail in Chapter XI. These effects were observed not only for natural BS but also for their synthetic analogs, which sometimes appeared to be very active. One of them, (22*S*,23*S*)-EBl, evoked the growth of leaves, increased

the weight of plants by 30-75%, and increased seed weight per plant by about 45% after treatment of string bean seedlings with a lanolin solution (Meudt *et al.*, 1983).

The numerous results that show the similarity in action of natural BS and their synthetic analogs, combined with the higher availability of the latter, have initiated extensive application of these analogs as model compounds in the investigations of the mode of action of BS. The present knowledge about the occurrence of BS in the plant kingdom is not exhaustive and some, nowadays still "nonnatural", BS might be found in natural sources in the future. Such a situation took place with EBl, described and investigated first as an accessible synthetic analog of Bl (Thompson *et al.*, 1979) and then isolated from *Vicia faba* L. pollen (Ikekawa *et al.*, 1988). At the same time, by using available synthetic analogs as a model for natural BS, one should keep in mind the possible restrictions, which are connected with the dependence of the activity on variations in the structure.

The growth-promoting activity of BS usually takes place only after treatment of plants in the appropriate phase of development and within a certain concentration range, which is different for each kind of plant and type of BS. Such a result is probably caused by the influence of BS on the total plant hormone status. The ratio between some components in the hormonal spectrum could also be important as it is known for other phytohormones, in particular for cytokinins (Galston *et al.*, 1983).

Noncorrespondence between the actual conditions of treatment and the optimal conditions for treatment can result in an absence of response or in inhibition of growth and decrease of crop yield. An example of such a behavior is given by Luo (1986b), who investigated the influence of concentration and conditions of BS treatment on the growth and crop yield of soybean. It was shown that a concentration of 0.1 ppm Bl enhanced the growth and dry weight of plants, whereas an increase of the concentration up to 1 ppm resulted in some

delay of leaf growth and reduction of weight in comparison with the control. In another case an increase of crop yield was reached after treatment of the plants in the period prior to flowering, but when treatment was carried out after flowering, the crop yield decreased. Probably, these effects have stimulated studies on the applicability of Bl in agents for restriction of growth (Hata *et al.*, 1986). It is probable that a significant part of the contradictions in the literature on the influence of BS on plant growth and development is connected with the indicated peculiarities of the realization of their effects. That is why special attention should be paid to the planning and execution of experiments and to a detailed analysis of all conditions in order to obtain reliable and comparable results and arrive at correct conclusions.

A comparative analysis of the action of different BS in an early stage of plant growth and on further productive development, which was done recently using wheat cv. Chinese Spring, euploid, and 18 related ditelocentric lines (DT-lines) with fixed genetic differences as a model system, showed a complex picture of effects (Mazets, 1997). The marked lines of hexaploid soft summer wheat cv. Chinese Spring differ from the euploid by the absence of one of the arms in one pair of homologous chromosomes. Some of the tested genotypes were highly sensitive to the action of BS, some of them showed medium sensitivity, and

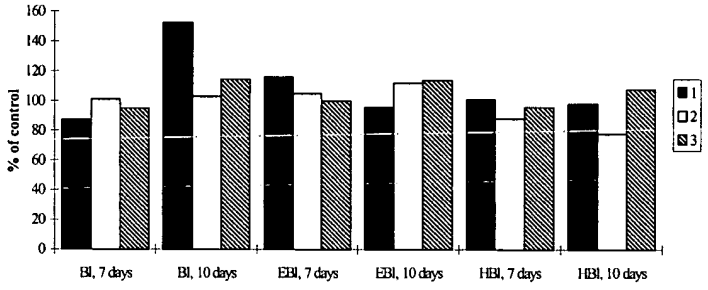

Fig. 35. Effect of BS on the fresh weight of 7-day-old and 10-day-old seedlings grown from seeds soaked in a 0.01 ppm solution of BS (1 - euploid, 2 - $5B^{L}$-line, and 3 - $5B^{S}$-line).

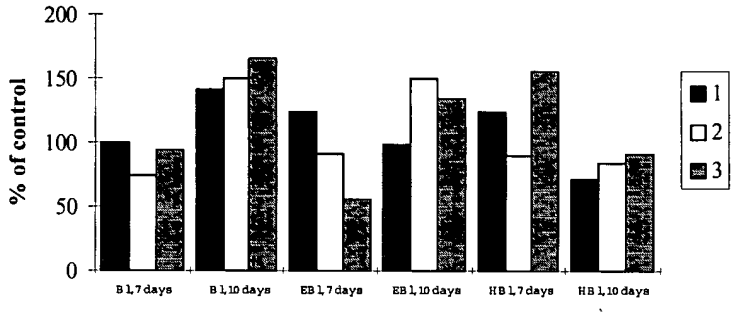

Fig. 36. Effect of BS on the fresh weight of roots of 7-day-old and 10-day-old wheat plants (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

others were not sensitive at all. The effect of BI, EBI, and HBI in two different stages of plant development in the euploid and in two DT-lines that are characterized by the absence of a short ($5B^L$) or a long arm ($5B^S$) in the chromosome 5B is shown in Fig. 35.

Although the data obtained for the treated plants are not much different in this period from the control, a high variability in plant response dependent on the structure of the BS and plant system was observed. Figure 36 illustrates the changes in root growth in the same plant system when BS were applied by soaking of the seeds with a 0.01 ppm solution of BS.

These data add another example to the previously described discrepancies in the literature data on root responses. Figure 36 shows a wide spectrum of effects within a short period of time. They strongly depend on the plant genotype and range from inhibition to stimulation. An interesting detail is the higher stimulation of the $5B^S$-line, which is characterized by the highest deficiency in the chromosome apparatus.

This tendency found in BS-influenced root growth became more obvious when the plant productivity was measured. Figures 37 and 38 show the data on two elements of crop structure in comparison with the control. These data were

obtained for two years, which had rather different weather conditions. Although the final effect of BS on the grain yield for these years is different and the ratio between the parameters has changed, in general a higher stimulation of the $5B^S$-line in comparison with the euploid and $5B^L$-line is seen. These data indicate that there is no clear correlation between the growth responses at early stages of plant development and the crop yield. They also lead to the conclusion that minor structural differences in BS, which usually are considered as unimportant for their activity, can be critical for the effect and the same can be said about the minor shifts in the genetic structure of the plant.

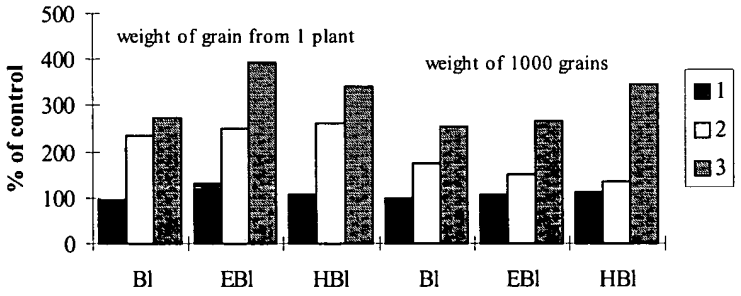

Fig. 37. Effect of BS on grain weight per plant and the weight of 1000 grains in 1991 (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

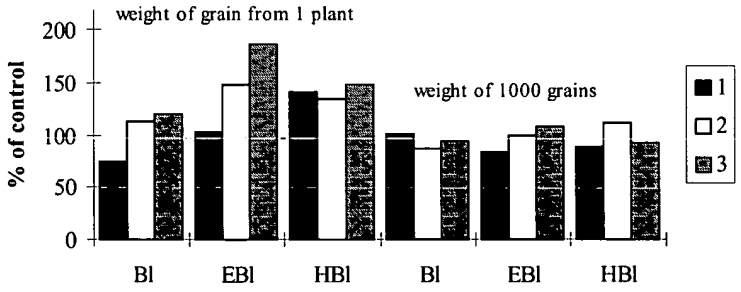

Fig. 38. Effect of BS on grain weight per plant and the weight of 1000 grains in 1995 (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

Additional confirmation of the complexity of the relation between the mode of application of BS and their effects on plant growth and development was obtained recently with new plant model systems and new methodological approaches. A characteristic influence of BS on plant development that depends on the time of BS application was found in investigations of the effects of EBl on large cranberry plants, *Oxycoccus macrocarpus* Pers. (Volodko and Zelenkevich, 1998). A special feature of the generative development of these plants is that the flower buds are formed on the top of uprights in the period from the second half of July to the first half of August in the year preceding the year of fruiting. The process of morphogenesis of the generative sphere continues till October and then it starts again in April. In May, when the average day temperature comes above the level of 5-7 °C, the development of the generative organs becomes visible. The flower bud starts to swell and then opens, and a new axial shoot with an inflorescence appears. As a rule, one top bud gives only one inflorescence with a variable amount of flowers (from 1 to 9-11, but usually 3-5). The 5-year-old plants were sprayed with a 10^{-5}% solution of EBl at a dose equivalent to 12.5 mg/ha at the beginning of August. Analysis of the crop parameters in the year of plant treatment showed no visible changes in comparison with the control. At the same time, the treatment acted efficiently on the development of the generative sphere, which at that time was in the beginning of its formation. In the year after treatment it was observed that in many plant buds more than one axial shoot with inflorescences appeared simultaneously from the top. The morphological structure and development of these shoots were not different from those of the control. On the inflorescences the normal buds were formed, opened, and flowered. The flowers were normally fertilized and gave ovaries. The percentage of top generative buds with this type of development was about 10-15% depending on the variety (Table XII). It should be pointed out that the amount of buds on such a shoot was the same as in the control.

TABLE XII

Effect of EBl on Cranberry Generative Development

Variety	Treat-ment	Number of generative uprights, % of total amount			
		1 Infloresc.	2 Infloresc.	3 Infloresc.	4 Infloresc. or more
Stevens	control	100.0	0.0	0.0	0.0
	EBl	84.6	7.6	4.3	3.5
Ben Lir	control	100.0	0.0	0.0	0.0
	EBl	88.8	5.7	3.1	2.4
Franklin	control	100.0	0.0	0.0	0.0
	EBl	89.1	6.0	3.2	1.7

An enhancement of the number of inflorescences was reflected in a larger crop yield of berries. Although the average weight of a berry was similar to that of the control, their larger amount was the reason for the increase of plant productivity by 9-15% (Table XIII).The treatment of plants with EBl did not affect the development of vegetative buds; in all shoots with vegetative buds only one new shoot was formed.

TABLE XIII

Effect of EBl on the Productivity of Cranberry Plants

Variety	Treat-ment	Number of berries from 1 m^2	Weight of one berry	Yield of berries, g/m^2	Crop increase	
					g/m^2	%
Stevens	control	2870	0.91	2583	-	-
	EBl	3343	0.89	2975	392	15.1
Ben Lir	control	2994	0.80	2395	-	-
	EBl	3321	0.81	2690	295	12.3
Franklin	control	3214	0.75	2410	-	-
	EBl	3519	0.75	2649	239	9.9

The discussed effect was observed only in the first year after plant treatment. The effect may be explained by a partial release of apical dominance in the generative organs after the application of EBl by regulation of the hormonal status in the apical part of the plant, which resulted in the enhancement of the number of inflorescences.

This conclusion is in agreement with the data obtained from the studies of the effect of EBl on the apical dominance in barley plants. Because this hormone-regulated phenomenon determines the character of interconnections between the shoots in cereal plants and strongly influences their productivity, an attempt to reduce it by the application of EBl has been done recently (Laman *et al.*, 1997; Vlasova *et al.*, 1998).

The final effect of BS is strictly dependent on the stage of plant development at the time of BS application; therefore the morphophysiological correlation between shoots in barley plants was studied for different times of EBl application, e.g., in the phase of full unfolding of the 2nd leaf, the 3rd leaf, and the 4th leaf in the main shoot. The best result in apical dominance release was achieved when the plants were treated with a dose that was equivalent to 50 mg of EBl per hectare in the phase of the 2nd leaf; when the main shoot apex becomes generative. The data were similar for two varieties of barley plants, cv. Prima Belarusi and cv. Visit. The final effect of EBl became apparent in the appearance of a larger amount of reproductive shoots and in a higher grain yield (Table XIV).

The observed effect is directly connected with a higher synchrony in the development of tillering shoots initiated by the application of BS. A high synchrony is important for the proportional distribution of assimilates in cereal plants and for a better grain production. The synchrony of shoot development was estimated for five varieties of barley plants treated with EBl in the phase of the 2nd leaf. The calculations were performed using a number of approaches (Paroda, 1971; Dahiya *et al.*, 1976; Faris and Klink, 1982) based on counting the

TABLE XIV

Effect of EBl on Tillering Shoot Formation and Crop Yield

Phase of development	Treatment	Number of productive shoots	Grain from one plant, g
two leaves	control	4.2	4.759
	EBl	6.0	5.372
three leaves	control	4.2	4.759
	EBl	4.9	4.049
four leaves	control	4.2	4.759
	EBl	3.9	3.551

number of days after planting till the development of the main shoot and the shoots of primary tillering had reached stage 50 (Zadoks *et al.*, 1974). These data were used for the calculation of the intraplant variance (σ^2), the regression coefficient (b_{xy}), the synchrony measure (SM), and the synchrony range (SR). The higher the synchrony of the development of the main and the side shoots is, the lower are the values of the mentioned parameters corresponding to it. As shown in Table XV, the treatment of plants with EBl significantly increased the synchrony of shoot development.

An additional quantitative characteristic of the ability of BS to stimulate growth processes in plants is illustrated by Fig. 39. It shows the reduction of phyllochrone value (Ph) for EBl-treated plants. This value is determined as the number of growing degree days (GDD) that are necessary for the unfolding of each next leaf. Ph and GDD are indicated for different stages of development in accordance with Haun (1973). Barley plants were treated at the 2nd leaf stage with EBl or gibberellic acid (GA).

TABLE XV

Effect of EBl on Synchrony of Plant Development

Variety	Treatment	Parameters of synchrony			
		SR	SM	b_{xy}	σ^2
		1995			
Prima Belarusi	control	21.5	31.4	6.8	10.1
	EBl	11.5	18.8	4.4	3.7
		1996			
Visit	control	7.0	16.4	2.2	11.8
	EBl	4.5	10.1	1.5	3.9
Lipen	control	11.1	18.3	3.5	22.5
	EBl	5.3	13.6	1.7	5.5
Maladzik	control	3.7	5.3	1.3	3.0
	EBl	3.5	4.5	1.0	2.3
		1997			
Visit	control	7.5	16.4	2.5	10.7
	EBl	3.9	8.4	1.3	2.8
Lipen	control	5.5	13.3	1.8	5.8
	EBl	3.5	6.3	1.2	2.5
Zazersky	control	6.8	12.2	2.3	9.4
	EBl	4.6	8.7	1.6	4.2

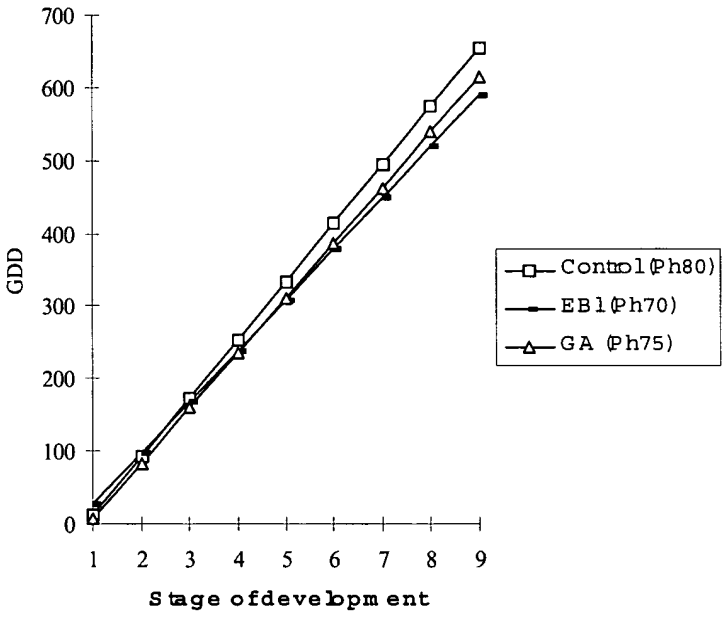

Fig. 39. The reduction of phyllochrone value (Ph) for EBl-treated plants.

A new illustration of the ability of BS to initiate a better realization of plant genome resource than usually takes place in normal plant development was found in studies of the effect of EBl on the ratio of morphotypes of barley plants that differed in the number of leaves in the main shoot (Laman *et al.*, 1997; Vlasova *et al.*, 1998). Treatment with EBl induced the development of plants with the maximum amount of leaves that is characteristic for the variety (Table XVI). For example, in the cv. Visit only 53% of the control plants came to full development with 9 leaves, which was the maximum number for this variety. After treatment with EBl, 100% of the plants showed full development. Similar effects were observed in the other two varieties.

In recent years several attempts to clarify the origin of the influence of BS on plant growth were undertaken. To explain the phenomenon of cell expansion under the action of BS, different hypotheses were developed and examined experimentally. It was found that the effect of BS is genetically determined and that BS are probably involved in all steps of cell growth regulation. Alterations in the mechanical properties of the cell walls (Tominaga *et al.*, 1994) and their relaxation and expansion may be regulated by BS *via* activation of specific genes controlling these processes (Clouse *et al.*, 1992; Wang, T.-W. *et al.*, 1993; Zurek and Clouse, 1994; Zurek *et al.*, 1994). Minor structural changes in plant tissues *via* the initiation of shifts in the orientation of microtubules within the cell followed by the corresponding shifts in the orientation of cellulose microfibrils are involved in the process of elongation and may be induced by BS (Mayumi and Shibaoka, 1995). The mechanistic aspects of BS-regulated plant growth will be discussed below.

TABLE XVI

Number of Leaves in the Main Shoot of Barley Plants Treated with EBl

Variety	Treatment	Number of plants, % of total amount			
		6 Leaves	7 Leaves	8 Leaves	9 Leaves
Visit	control	0	0	47	53
	EBl	0	0	0	100
Lipen	control	27	73	0	0
	EBl	0	80	20	0
Zazersky	control	0	0	46	54
	EBl	0	0	7	93

B. INTERACTION WITH OTHER PHYTOHORMONES

One of the first questions evoked by the determination of the high plant growth promoting activity of BS was the behavior of these new phytohormones in the tests that were supposed to be specific for auxins, gibberellins, and cytokinins. Also the mutual interaction with other plant hormones was a subject of interest. In tests (Yopp *et al.*, 1981) on elongation of etiolated maize mesocotyl segments, elongation of pea epicotyl and azuki bean epicotyl segments, and retardation of hook opening of etiolated bean hypocotyls, a similarity in the effects of Bl and IAA was shown. However, in a number of other tests such as inhibition of cress root elongation and inhibition of lateral bud growth on decapitated pea plants, Bl appeared to be inactive. It did not promote, but elicited retardation, of mung bean hypocotyl rooting.

Strong synergism between auxins and BS was observed by several investigators (Yopp *et al.*, 1981; Takeno and Pharis, 1982; Arteca *et al.*, 1983; Meudt and Thompson, 1983; Choi, C.-D. *et al.*, 1990b; Cao and Chen, 1995). It proved to be dependent on the sequence of treatments of plants and it appeared only in cases where BS treatment preceded the auxin treatment. Typical auxin inhibitors, such as (*p*-chlorophenoxy)isobutyric acid, suppressed the BS action and the joint influence of both hormones. One of the most sensitive tests for BS is based on the enhancement of the auxin-induced curvature of the first bean internode. The response to Bl of vertically placed hypocotyls was dependent on exogenous auxin. In contrast, the response of gravistimulated sections to Bl was observed in the absence of auxin (Meudt, 1987). The assumption (Takeno and Pharis, 1982) about the mediating of the effects of BS in plants *via* an increase of the auxin level was checked and it was proposed that Bl did not depend on auxin as a mediator but could interact with it in a complex manner (Sasse, 1990, 1991a). This is in agreement with previous data obtained with EBl, which did

not affect the level of IAA in plant tissue, the rate of IAA transport, or its metabolism (Cohen and Meudt, 1983).

Further evidence for synergism between BS and auxin was obtained in studies of ethylene production by etiolated mung bean (*Vigna radiata*) hypocotyl segments (Arteca *et al.*, 1983, 1985; Arteca, 1984; Schlagnhaufer *et al.*, 1984a; Arteca and Schlagnhaufer, 1984; Tsai and Arteca, 1985; Schlagnhaufer and Arteca, 1985b). The combined treatment of plants with Bl and IAA caused a production of 43.6 nl/h ethylene; in the control the corresponding value was 0.41 nl/h. After separate treatments, these values were 10.5 nl/h for Bl and 17 nl/h for IAA. It was shown that Bl and IAA both affect ethylene production in the stage of transformation of *S*-adenosylmethionine to aminocyclopropane-1-carboxylic acid (Schlagnhaufer *et al.*, 1984b) and that their effects on ethylene production could be inhibited by the application of fusicoccin (Arteca *et al.*, 1988b), (aminooxy)acetic acid (Arteca *et al.*, 1988a), or some other auxin antagonists (Arteca *et al.*, 1991). Increase in ethylene production was found in rice plants treated several days before ear formation with Bl or EBl together with plant growth retardants, when they were controlled at the milk-ripe stage (Saka *et al.*, 1992). When Bl and ethylene were used in combination, an additive growth-stimulatory effect was observed in rice coleoptiles (Zhou, A.-Q., 1987).

The cooperative action of Bl with gibberellin A_3 was also investigated (Gregory and Mandava, 1982). The treatment of young mung bean seedlings with both growth promoters separately resulted in elongation of epicotyls, but BS showed this effect at a lower concentration and gave higher elongation (up to 1000%). Also epinastic curvature of the epicotyls and petioles was observed for BS whereas this was not the case for gibberellic acid. Similar behavior was noticed for tomato plants (Schlagnhaufer and Arteca, 1985a,c). Treatment with BS and GA_3 together did not show any synergism, but the two hormones acted in an additive manner (Gregory and Mandava, 1982). A retardant of the elongation effect for GA_3, e.g., ancymidol [α-cyclopropyl-α-(4-methoxyphenyl)pyrimidine-

methanol], did not interfere with the BS response, and this fact was taken as evidence for an independent role of both phytohormones in plants. A similar conclusion can be drawn from experiments on pea segments (Sasse, 1988) and rice root cuttings (Radi and Maeda, 1988). BS were reported to affect to some extent the endogenous gibberellin level (Skorobogatova, 1991).

It was shown that BS changed the composition of cytokinins in plant leaves (Kislin and Semicheva, 1991; Kozik and Kislin, 1991). Treatment of barley plants with EBl in a dose of 10-50 mg/ha resulted in a 6-12-fold increase of zeatin-riboside (ZR); a reduction of zeatin was noticed simultaneously. Dihydrozeatin-riboside, dihydrozeatin, and isopentyladenine were found in leaves of treated plants, but not in the control plants.

Inconsistent data on the influence of BS on the level of abscisic acid (ABA) were obtained (Eun *et al.*, 1989; Kozik and Kislin, 1991; Korablyova and Sukhova, 1991; Kuraishi *et al.*, 1991). Thus, treatment of barley plants with EBl in the beginning of flowering resulted in a 10-fold decrease of endogenous ABA analyzed in the milk phase in comparison with the control (Kozik and Kislin, 1991; Kurapov *et al.*, 1992). In the range of the tested doses (10-50 mg/ha) no dose-effect relationship was found; evidently, the threshold of sensitivity was lower than 10 mg/ha. When barley plants were treated in the booting phase, a significant increase was observed only for IAA (Kurapov, 1996). A simultaneous increase of the levels of IAA and ABA was observed in *Gossypium hirsutum* plants after their treatment with Bl (Luo *et al.*, 1988), and GA_3 and ABA in cucumber hypocotyls after treatment with EBl (Xu *et al.*, 1990). An increase of the ABA level was found in potato tubers treated with EBl before storage (Korablyova and Sukhova, 1991).

A comparative investigation of the changes in levels of IAA and ABA and in the evolution of ethylene in squash hypocotyls after treatment with Bl showed its stimulative effect on the levels of IAA and a tendency to decrease the level of

ABA. The production of ethylene was not much influenced by Bl in these experiments (Eun *et al.*, 1989).

An analysis of shifts in the content of three phytohormones (IAA, ABA, and ZR) under the action of BS showed a significant difference in the effects of Bl, EBl, and HBl in wheat cv. Chinese Spring and its DT-lines (Mazets, 1997). The data obtained for two of them, the $5B^L$- and $5B^S$-lines, and for the euploid are shown in Fig. 40. The 10-day-old wheat plants grown from seeds treated with BS (soaked in 0.01 ppm solution) were analyzed and compared with an untreated control.

These data show the high specificity of the interaction of each BS with the genotypes. Strong stimulation of the ABA level was observed for the plants of the $5B^L$-line that were obtained from seeds treated with EBl or HBl (850% and 680% of control, respectively). The plants of the $5B^S$-line also gave a good response (363% and 298% of control, respectively). A reasonable increase of the IAA level was registered only for EBl in the euploid (187% of the control), and an increase of the ZR level was found in the same plants when treated with HBl (180% of control). A number of these measurements showed a suppression in phytohormone accumulation in this period of plant development under the action of BS.

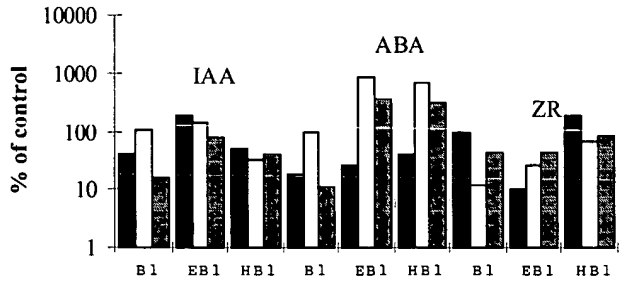

Fig. 40. Effects of BS on content of different phytohormones in wheat cv. Chinese Spring and in its DT-lines (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

The evidence mentioned above about the influence of BS on the function and content of other hormones in plants evoked the suggestion that the observed macroeffects could be mediated in this way. Similar considerations created the basis for a hypothesis about the managing role of BS with respect to other hormones (Takematsu, 1988), but this hypothesis still requires confirmation. As for the place of BS in the total hormone spectrum, it was shown that the maximum sensitivity to EBl during plant development occurred after that to GA and cytokinin and began before that for auxin in isolated wheat coleoptiles. In dwarf pea segments, the maximum sensitivity to BS laid between those of GA and auxin (Sasse, 1985).

C. EFFECT ON CELL MEMBRANES

1. H^+-Pump Activation and Electrical Properties

The biochemical changes that take place in the cell membranes play a key role in the growth responses of plants to the application of exogenous BS. Membranes also define, to a high degree, many other aspects of the physiological action of BS. The data obtained to date show that BS are capable of influencing the electrical properties of membranes, their permeability, and the structure, stability, and activity of membrane enzymes. One of these effects, connected with the activation of the membrane-bound proton pump, showed similarity in the action of BS and IAA. This fact allows one to assume that the initial stage of cell elongation in both cases is connected with loosening of the cell wall as a result of its acidification by H^+ ions and activation of polysaccharide hydrolases. Increase of the cell size takes place as a consequence of reduction of the cell wall resistance to the pressure of the intracellular contents followed by water uptake.

In an early study of BS-stimulated acid secretion, a pronounced effect of EBl in azuki bean epicotyls, in shoots, and in apical and subapical root segments of maize at concentrations of 10^{-7}-10^{-5} M was shown (Cerana *et al.*, 1983a,b; Romani *et al.*, 1983). A significant stimulation of growth associated with increased acid secretion was accompanied by an early hyperpolarization of the transmembrane electric potential. These effects of BS, and those of IAA, were suppressed by inhibitors of RNA and protein synthesis (Cerana *et al.*, 1983a). BS-induced acid secretion was enhanced by the presence of K^+ ions in the medium (Romani *et al.*, 1983). Additivity of the BS and IAA effects was found for azuki bean epicotyls (Cerana *et al.*, 1983a). A difference in the behavior of two phytohormones under some experimental conditions (Romani *et al.*, 1983) suggested different pathways for the action of BS and IAA on the proton pump. The suggestion that BS affects the cytoplasmic metabolism through acid secretion is based on data on the enhancement of malate content and rate of CO_2 fixation in maize root segments together with activation of H^+ extrusion. Study of the same segments under conditions when IAA decreased acid secretion, malate content, and rate of dark CO_2 fixation led the authors to the conclusion that activation and inhibition of the rate of operation of the H^+ pump was connected with a change in phosphoenolpyruvate carboxylase activity (Cerana *et al.*, 1983b).

BS-induced acid secretion showed a close correlation with the electrical parameters of the membranes and with BS-stimulated plant growth. Quite characteristic in this respect is the influence of Bl and EBl on growth and proton extrusion in the green alga *Chlorella vulgaris* (Baiguz and Czerpak, 1996). Although some of the natural BS, including EBl, have been found in *Chlorophyta*, there are few data on the biochemical effects of exogenous BS in algae. The results indicate an intense stimulation of growth of the algae under the influence of BS at concentrations of 10^{-15}-10^{-8} M. Bl showed slightly more activity in comparison to EBl. Between the 12th and the 36th hour of the

experiment BS induced an intense growth of the alga that was two to three times higher than in the control. The intensity of H^+ secretion correlated with the dynamics of cell growth and was dependent on the concentration of BS. A slightly higher activity of Bl as H^+ secretion stimulant was observed here also.

As already mentioned above, many studies on the biological activity of BS were based on the employment of easily available nonnatural BS analogs such as (22*S*,23*S*)-HBl. When only these analogs are used to find out how the real hormone is functioning in plants, there is a great probability that the outcome will not be correct. The structural incompatibility of the analog with the putative hormone receptor together with the multifunctionality of steroids in plants, which usually will cause some plant response, easily could lead to false conclusions. Experiments with nonnatural BS analogs are, however, very useful for determination of the experimental boundaries of BS test systems.

An example of this approach is the study on proton extrusion and electric cell properties in *Egeria* leaf cells under the action of (22*S*,23*S*)-HBl and 2α,3α-dihydroxy-5α-stigmast-22-en-6-one, two BS analogs with a different level of similarity to Bl (Dahse *et al.*, 1990). Their effects were compared with the activity of stigmasterol and the nonsteroidal plant growth regulator fusicoccin and the structural requirements for their action were estimated. BS analogs and fusicoccin in the light showed similar effects in hyperpolarization and proton extrusion whereas stigmasterol was less effective. In darkness, the effects of the three steroids were comparable. The fact that all steroids caused acidification of the medium indicates that no special structural requirements are needed for the stimulation of acid secretion. The ability of all steroids to hyperpolarize the cell potential in accordance with the theoretical view on the components contributing to the plasma membrane potential strongly suggests the stimulation of an electrogenic pump. Rather characteristic changes in the membrane potentials caused by different steroids, in comparison with the less specific data on medium acidification at the same conditions, suggest that the membrane potential reflects

proton pumping more sensitively than medium acidification. In contrast to fusicoccin, the effect of the steroids on the membrane potential and on medium acidification was reversible. A different behavior of (22*S*,23*S*)-HBl and fusicoccin was observed in an experiment on Fe^{3+} reduction and also on sucrose and amino acid uptake. These facts indicate that the mechanism of steroid action on proton pumping is different from that of IAA and fusicoccin. These data together with earlier results (Cerana *et al.*, 1984) on the plasmalemma proton pump activation by different sterols show that the most probable mode of action of BS is an indirect effect on the lipid environment of the enzyme; however, shifts in metabolic processes cannot be excluded.

New data on the effect of BS on the membrane properties in root cells have been obtained from studies of the action of EBl on young barley and triticale plants (Kalituho *et al.*, 1997b; Kalituho and Kabashnikova, 1998). The seeds were soaked in an EBl solution (10^{-8} M) for 6 h, then grown in tap water for 4 days, and cultivated in a solution of 10^{-3} M KCl + 10^{-4} M $CaCl_2$. Investigation of 7-day-old plants showed a significant decrease of the H^+ concentration in culture medium in comparison with the control (Table XVII). No essential changes in fresh weight or in growth of roots, coleoptiles, and the first leaves were observed except for a significant root elongation in one of the two varieties of the triticale plants (cv. Dar Belarusi).

Along with the diminishing of the total H^+ concentration ($[H^+]$), the H^+ concentration related to the root biomass ($[CH^+]$) was decreased also. These data, obtained from experiments with intact seedlings, are opposite to the data previously discussed on the activation of acid secretion by roots that were treated with EBl. The main reason for this difference could be the mode of treatment of the plants, application of EBl by addition to the nutrient medium or by soaking the seeds. The last variant reflects a long term effect of EBl: from the moment when the seeds were treated to a time after 7-8 days when the plants were studied for the results.

TABLE XVII

Effect of Epibrassinolide on Growth and H^+-Pump Activity

Treatment	Length, % of control			Fresh weight, % of control		pH	$[H^+]$		$[CH^+]$	
	Root	Coleop-tile	First leaf	Root	Leaf+Co-leoptile		M/g	% of control	M/g	% of control
Barley, cv. Honar										
control	100	100	100	100	100	6.12	$0.75 \cdot 10^{-6}$	100	$0.80 \cdot 10^{-6}$	100
EBl, 10^{-8} M	104	100	101	103	101	6.19	$0.66 \cdot 10^{-6}$	88	$0.69 \cdot 10^{-6}$	86
Winter triticale, cv. Malno										
control	100	100	100	100	100	4.70	$0.20 \cdot 10^{-6}$	100	$0.16 \cdot 10^{-4}$	100
EBl, 10^{-8} M	105	101	104	93	100	4.86	$0.13 \cdot 10^{-6}$	65	$0.12 \cdot 10^{-4}$	75
Winter triticale, cv. Dar Belarusi										
control	100	100	100	100	100	4.52	$0.50 \cdot 10^{-6}$	100	$0.43 \cdot 10^{-4}$	100
EBl, 10^{-8} M	133	100	102	97	97	5.04	$0.11 \cdot 10^{-6}$	22	$0.10 \cdot 10^{-4}$	23

The obtained data can be considered as an additional indirect confirmation of the existence of a concerted mechanism between BS-initiated growth and acid secretion. Rather promising for further clarification of this relation is an estimation of proton pump activity under the conditions of growth inhibition, which are well documented in the literature (Roddick and Guan, 1991).

Because the activity of the proton pump is closely related to the transmembrane transport of different ions and of organic substances, an attempt to estimate the functioning of membranes has been done based on the excretion by cells of some neutral metabolites (Kalituho and Kabashnikova, 1998). Some products of the nucleotide metabolism have a characteristic UV absorption at 260 nm and this wavelength was used to follow spectrophotometrically the changes in excretion of these metabolites. Usually this excretion is rather low under normal conditions but becomes much higher under stress conditions. The results summarized in Table XVIII show that at 20 °C and under heat stress (50 °C) the excretion of these metabolites was lower for the plants grown from treated seeds than in the control.

Similar to the results on the deactivation of the proton pump, these data suggest a stabilizing effect of EBl on cells *via* modification of the plasma

TABLE XVIII

Effect of EBl on the Secretion of Nucleotide Metabolites

Treatment	Leaves				Roots			
	t=20 °C		t=50 °C		t=20 °C		t=50 °C	
	D_{260}	% of control	D_{260}	% of control	D_{260}	% of control	D_{260}	% of control
control	0.099	100	0.114	100	0.138	100	0.176	100
EBl	0.096	96	0.106	93	0.094	68	0.144	81

membrane activity during plant development. Such an effect could play a role in the maintenance of plant cell homeostasis and contribute to the adaptation mechanism. This also might be an explanation for the antistress action of BS in plants.

A comparison of the data in Table XVIII on the effects of EBl on the roots and on the first leaf leads to the conclusion that the stabilizing effect in the leaves is much weaker than in the roots, at least in this stage of plant development. The tissue specificity of EBl action is reflected also in the metabolite excretion in the roots and in the leaves under heat stress. In the roots the relative level of BS inhibition was lower under stress than in normal conditions (32% and 19%, respectively), and in the leaves the degree of BS-affected excretion inhibition was higher under stress conditions (4% and 7%, respectively).

Of considerable interest for better understanding the correlation between growth processes and changes in the activity of membrane enzymes influenced by BS are the results of studies of the effect of EBl on growth, acid secretion, and H^+-ATPase activity in roots of maize (Palladina and Simchuk, 1993; Palladina *et al.*, 1995). Hydroponically grown, 8-day-old maize seedlings were examined for changes produced by the application of EBl *via* three different ways: (1) soaking the seeds in an EBl solution for 24 h; (2) exposure the roots in a solution containing EBl; (3) spraying the upper part of the seedlings with an EBl solution.

A 24-h exposure of the 7-day-old seedlings in EBl solution (10^{-7}-10^{-9} M) increased the mass of roots, acid secretion, and K^+ uptake, which was in agreement with the earlier results of Cerana *et al.* (1983a) discussed above; most efficient was the concentration of 10^{-7} M EBl.. A dependence of the acid secretion on the functioning H^+ pump was illustrated by the inhibition of medium acidification in the presence of orthovanadate, an inhibitor of transport H^+-

ATPase, the enzyme that is responsible for the functioning H^+ pump in plasma membranes.

An attempt was made to estimate the effect of EBl on the activity of H^+-ATPase in plasma membrane preparations obtained from maize roots. It was found that all three different treatments with EBl *in vivo* increased the H^+-ATPase activity. A kinetic analysis showed that the application of EBl decreased the K_M value but significantly increased V_{max}. In *in vitro* experiments with plasma membranes, EBl showed no effect on the H^+-ATPase activity within an interval of concentrations from 10^{-7} to 10^{-9} M at pH 6.5. On the contrary, all the employed concentrations of EBl strongly inhibited of enzyme activity at pH 7 and pH 7.5.

A difference in the effects of EBl on the H^+-ATPase activities of plasmalemma and cytoplasmic components was found in 5-day-old seedlings of buckwheat (*Fagopyrum esculentum* Moench.), where total enhancement of the enzyme activity was fully controlled by cytoplasmic components and took place along with the enzyme activity decrease in the plasmalemma (Deeva *et al.*, 1996a).

The data of the studies (Palladina and Simchuk, 1993; Palladina *et al.*, 1996) indicated a possible role of BS-induced changes in the lipid environment of the proton pump protein as one of the mechanisms of BS control of the H^+-pump activity. It is well known that sterols play an important role in the structural organization of plasma membranes; therefore the effect of EBl on the sterol compositions and their quantity in membranes was investigated. It was shown that exposure the maize roots in a 10^{-7} M EBl solution led to a diminution of the sterol/phospholipid and sterol/protein ratios. Similar changes also took place with seedlings grown from EBl-treated seeds. Some variation in the contents of different phytosterols was also found.

This result logically leads to the detailed analysis of the BS-influenced changes in membrane composition, which can be considered to be an important element in the mode of action of BS.

2. Effect on Chemical Composition. Membrane-Protective Action

The BS-initiated changes in the phytosterol composition of membranes discussed above were mainly connected with the ratio of three sterols: cholesterol, sitosterol, and stigmasterol. The first and second ones have a saturated side chain, and the third one is the main plant sterol with an unsaturated side chain. In treated plants the contents of stigmasterol was increased, and the contents of sitosterol and cholesterol were decreased. In the case of cholesterol the decrease was about 50% in comparison with the control. The reduction of the total sterol content may be expected if there is a similarity in sterol biosynthesis regulation in plants and in mammals. In such a case, a mechanism in which 3-hydroxy-3-methylglutaryl coenzyme A reductase could be blocked *via* binding of oxygenated steroids, such as BS, with a cell component similar to the oxysterol receptor of mammals (Zeelen, 1990) has to be present in plants.

Along with the changes in sterol content, the alteration in protein and phospholipid components is very important in membranes, due to the direct connection of these parameters with the functional properties of membranes. Especially the chemical nature of the fatty acid composition of membranes is an important factor in the regulation of permeability because of their influence on the surface properties of phospholipids, on lipid-protein and lipid-lipid interactions, and on the activity of lipolytic enzymes.

The early findings on changes in fatty acid composition of membrane lipids showed clearly an enhancement of the unsaturated acids in comparison with the saturated ones after BS treatment (Zhao and Wu, 1990; Katsumi, 1991). Further

investigation with new plants and experimental conditions confirmed the previous results and gave a better insight into the phenomenon (Vedeneev *et al.*, 1997b).

Two varieties of barley plants, cv. Roland (R) and cv. Zazersky (Z), and their isoplasmatic lines R(Z), bearing in cells the nucleus of R and the cytoplasm of Z, and Z(R), bearing in cells the nucleus of Z and the cytoplasm of R, were treated with EBl solution (0.01 ppm) in the beginning of the tillering phase. After 72 h plant leaves were investigated for their fatty acid composition using GC analysis. It was found that the application of EBl significantly affected the content of fatty acids and resulted in an enhancement of the unsaturated acids and in a reduction of the saturated ones (Table XIX).

The Zazersky variety, which initially had the lowest unsaturation index (I) {I $=([C_{16:1}] + [C_{18:1}] + 2[C_{18:2}] + 3[C_{18:3}])/([C_{16:0}] + [C_{18:0}] + [C_{20:0}])$}, showed the highest changes after EBl treatment; the unsaturation degree became about three

TABLE XIX

Effect of EBl on the Fatty Acid Composition in the Fraction of Saponifiable Lipids for Different Genotypes of Barley Plants (72 h after Treatment), %

Plants	Treat-ment	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Satur/ unsatur	Index
R	control	4.14	0.29	1.13	1.46	92.02	0.06	0.056	35.28
	EBl	2.48	0.24	0.36	1.78	94.88	0.26	0.029	67.80
Z	control	7.55	0.40	1.79	5.08	84.76	0.42	0.103	18.87
	EBl	2.93	0.15	0.47	2.10	94.19	0.16	0.035	56.21
R(Z)	control	4.32	0.57	0.54	1.99	92.46	0.12	0.051	38.56
	EBl	2.49	0.27	0.25	1.50	95.38	0.11	0.028	70.39
Z(R)	control	3.80	0.70	0.60	1.38	93.40	0.12	0.046	43.00
	EBl	3.09	0.06	0.51	1.38	94.48	0.48	0.037	53.29

times higher. The weakest response was exhibited by the cytoplasmic line Z(R), which already had the highest unsaturation degree in the control. The Roland variety and the cytoplasmic line bearing its nucleus had quite similar parameters. This experimental approach gave no clear indications for the role of the nuclear or cytoplasmic genome in the mediation of the effect of EBl. The obtained data do not allow one to exclude a short-distance mechanism, implying a regulation by EBl of some membrane enzymes that are responsible for the biotransformation of fatty acids.

The shifts in composition of fatty acids are closely connected with the changes of lipid content and with the dynamics of lipid peroxidation, two additional parameters which are usually used for the specification of the status of the membrane. The influence of EBl on the lipid content in barley plants (cv. Roland) showed that all treatments gave a pronounced enhancement of total lipids. The data for a 4-day period in the tillering phase are shown in Table XX.

Application of EBl by spraying gave a higher effect than treatment of the seeds, and the combination of both treatments gave an efficiency similar to that of spraying alone. The observed effect of lipid increase was rather stable and

TABLE XX

Effect of EBl on the Lipid Content in Barley Plants

Treatment	mg/g of fresh weight					mg/g of dry weight				
	time, days					time, days				
	0	1	2	3	4	0	1	2	3	4
control	12.5	12.2	12.0	11.5	12.5	68.3	67.0	66.0	65.3	66.5
EBl, 0.01 ppm (seeds)	12.0	12.5	14.2	14.0	14.2	70.2	72.8	75.0	77.8	78.9
EBl, 0.01 ppm (spraying)	12.5	12.5	15.5	16.0	17.1	68.7	70.2	92.7	88.9	92.0

was preserved in the plants for a longer time.

A characteristic of the influence of BS on the membrane lipid components is not complete without information about changes in the fractions of saponifiable (fatty acid lipids) and unsaponifiable (sterols, glycosides, terpenoids, etc.) lipids because of their importance for the membrane fluidity. The data of Table XXI illustrate the changes of these components in four genotypes of barley plants under the influence of EBl, applied by spraying at a concentration of 0.01 ppm in the tillering phase.

At first glance, a diminution of the total content of lipids is rather unexpected, but taking into account that the total lipid fraction (Table XX) also included a growing amount of plant pigments, mainly chlorophyll, that are not present in the fractions of saponifiable and unsaponifiable lipids, the data of Table XXI become understandable. The most important result shown here is the

TABLE XXI

Effect of EBl on Saponifiable and Unsaponifiable Lipids in Different Genotypes of Barley Plants

Plants	Treatment	Content of lipids		
		Both fractions, mg/g of fresh weight	Saponifiable, %	Unsaponifiable, %
Z	control	8.18	49.51	50.49
	EBl	5.58	69.53	30.47
Z(R)	control	7.20	50.13	49.87
	EBl	6.24	58.33	41.67
R	control	9.42	68.79	31.21
	EBl	8.58	75.29	24.71
R(Z)	control	9.10	39.56	60.44
	EBl	6.96	48.85	51.15

pronounced change in the ratio of the two groups of lipids, reflecting a significant increase of the saponifiable lipids, which results in an increase of the membrane fluidity.

The dynamics of lipid peroxidation in cell membranes was studied (Vedeneev and Deeva, 1997) using the accumulation of malonic dialdehyde as the end product of the lipid peroxidation in plants (Table XXII).

The plants were sprayed with a 0.01 ppm solution of EBl in the tillering phase and then monitored for 4 days after the treatment. A significant retardation of the malonic dialdehyde accumulation was found starting from the first day after EBl application.

Since the chemical composition of the membranes is an important factor affecting the behavior of plants in stress conditions, further studies were carried out to clarify the relationship between higher stress resistance of BS-stimulated plants and the shifts in the membrane chemical composition. The peroxidation of

TABLE XXII

Effect of EBl on Lipid Peroxidation in Barley Plants (Malonic Dialdehyde Content, μM/g of Fresh Weight)

Plants	Treat-ment	Exposure, days				
		0	1	2	3	4
Z	control	33.53	42.05	28.21	58.55	50.03
	EBl	33.53	39.39	26.61	55.04	49.50
Z(R)	control	31.40	45.25	35.96	82.50	69.19
	EBl	31.40	33.00	25.02	63.34	50.56
R	control	44.18	44.71	46.57	48.97	63.87
	EBl	44.18	43.11	43.11	43.65	44.18
R(Z)	control	53.22	53.22	58.54	53.22	61.21
	EBl	53.22	43.11	46.84	44.71	45.24

lipid components of membranes occurs normally as part of the lipid catabolism. In stress conditions destructive processes in membranes become more intense, and this phenomenon is considered to be connected with lipid peroxidation. Taking into account the stress-protecting properties of BS, the lipid peroxidation was chosen as an approach for better understanding the mode of action of BS on cell membranes in different conditions (Ershova and Khripach, 1996).

The oxidative lipid degradation influenced by EBl was studied in 2-week-old pea seedlings (*Pisum sativum* L., cv. Ramonskii 77) at normal aeration, under oxygen deficiency, and in a CO_2-enriched atmosphere. The two last models are known to activate the degradation of phospholipids in biological membranes. Kinetin was used as a comparison because of its ability to maintain the elevated level of phospholipid desaturation under the applied conditions. Both EBl and the kinetin solution were introduced into the shoots by transpiration flow in the darkness. As criteria for the degree of degradation, the contents of products of lipid peroxidation, such as conjugated dienoic acids and malonic dialdehyde, were determined. The content of conjugated dienoic acids was shown to decrease in seedlings treated with EBl, and this effect was stronger than the lipid peroxidation inhibition caused by kinetin (Fig. 41). The inhibition was more

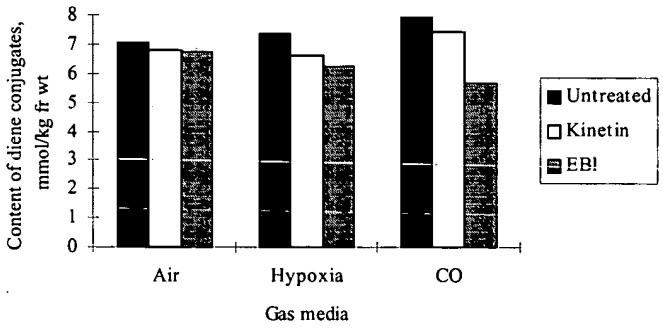

Fig. 41. Conjugated dienoic fatty acid content in pea seedlings treated with EBl or kinetin in different gas media.

efficient in plants under hypoxia or in the CO_2-enriched atmosphere, and the content of conjugated dienes for these cases was lower in treated plants by 13 and 21%, respectively, in comparison with air-grown seedlings.

The accumulation of malonic dialdehyde in plants in normal conditions was considerably decreased by EBl but not by kinetin (Fig. 42). Both hypoxia and elevated CO_2 inhibited malonic dialdehyde formation. Under hypoxia, this inhibition was higher when the seedlings were treated with the phytohormones, but in the CO_2-enriched atmosphere EBl did not show such an effect.

Taking into account the data (Leshem, 1984) on the lipoxygenase blocking mechanism of kinetin action, a similar influence on the lipoxygenase activity could be assumed to explain the mode of action of EBl, because its direct antioxidative activity is less probable. Finally, the inhibition of the lipid peroxidation by EBl contributes to a better maintenance and stability of biomembranes, and this is probably one of the ways in which the stress resistance of plants can be improved by BS.

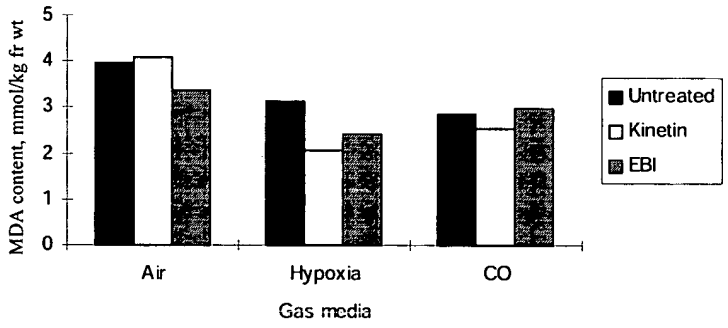

Fig. 42. Content of MDA in pea seedlings treated with kinetin or EBl in different gas media.

3. Membrane Permeability and Transport

The plasma membrane plays an important role among the different membrane structures of the cell because of its location as a peripheral external surface, bordering the cell content from the environment. Along with this mechanical barrier function, there are two other important functions of the plasma membranes: (1) providing conditions for exchange of information with the environment, mainly by reception and transmission of chemical signals in both directions, and (2) maintaining the cell homeostasis *via* the transport of ions and biologically important substances.

These functions are strongly related to the membrane bilayer permeability and the status of the membrane phospholipid matrix. As shown above, the latter can be regulated efficiently by BS.

The effect of BS on cell membrane permeability and transport has been reported by several groups. Some of the data were mentioned above in the discussion about the H^+-pump regulation and proton transport. Investigations on their correlation with transport and assimilate uptake by plant tissues showed that these processes are closely interconnected (Dahse *et al.*, 1990, 1991). Treatment of *Egeria densa* plants with (22*S*,23*S*)-HBl, along with hyperpolarization of the plasma membrane in light and in dark conditions, promoted the uptake of ^{14}C-labeled sucrose by the leaf cells. The changes in uptake rate for plants treated with 1 μM (22*S*,23*S*)-HBl were 110 and 121% compared to untreated controls for the light and for the dark, respectively. When a 10 μM concentration of (22*S*,23*S*)-HBl was applied, the corresponding results were 118 and 129% compared to the control. These values were even higher than that of fusicoccin, a known initiator of similar effects. (22*S*,23*S*)-HBl also stimulated the uptake of α-aminoisobutyric acid. At the most efficient concentration (0.1 μM) its uptake rate was 138 and 127% compared to an untreated control in the light and in the dark, respectively.

With respect to stimulation of the membrane transport of amino acids, it should be pointed out that a similar effect of human steroidal hormones, in particular glucocorticoids, on human cells has been reported in the literature (Sergeev, 1984).

This and other similarities between BS and mammalian steroids suggest that it may be useful to look a bit further than just to plants alone to gain knowledge about their mode of action. This means that in the study of a new BS phenomenon in plants, a look at the analogous situation with steroids in mammals or in insects might be more fruitful than the traditional comparison with effects of other plant hormones. Nevertheless, the traditional approach is also necessary because the role of BS in the total hormonal spectrum is not fully clarified yet.

An important aspect of BS-regulated transport in plants has been found in studies on the partioning of ^{14}C-labeled photosynthates in *Vicia faba* under the action of (22*S*,23*S*)-HBl (Petzold *et al.*, 1992). This compound, when applied exogenously to the source leaves, was found to increase the retention of [^{14}C]sucrose and to activate the uptake in discs of the source leaves during a 4-h treatment. IAA and GA_3 showed similar behavior in such experiments. A comparison of the treated plants after 24 h revealed the differences between the effects of the applied phytohormones. Only (22*S*,23*S*)-HBl and GA_3 significantly enhanced the transport of a model assimilate to the apical sink region. The hormone-activated sucrose uptake is probably caused by modification of the H^+-ATPase activity, which was illustrated by enhanced V_{max} values for sucrose uptake. The measured alteration in the kinetic properties of the uptake system correlates with the rapid effect (during 1 h) of BS on sucrose uptake in leaf discs. This work provides further support for hypothesis on the interrelation between the plasma membrane proton pump, energetization the proton-sucrose-coordinated transport, the sucrose uptake, and the phloem loading in leaf tissues.

A supposition about BS activation of phloem transport *via* initial promotion of the H^+-pump stimulated investigations of the effect of EBl on the dynamics of glucose accumulation in the organs of winter wheat (cv. Mironovskaya 808), barley plants (cv. Zazersky), and potatoes (cv. Nevskii) (Kurapov *et al.*, 1995). Application of [^{14}C]glucose on the leaves of control and EBl-treated plants was used to follow the assimilate transport. Treatment of cereal plants with EBl in the flowering stage activated the transport of labeled substance into the ear; the maximal intensity of this process was observed after 24 h. The application of EBl to potato plants activated the accumulation of the label in the lower part of the plants and in the tubers, and its content grew during the month.

Practically promising results concerning the regulation by BS of the cell permeability for ions were obtained recently using different model systems in experiments on the absorption of heavy metals and radionuclides by plants. The accumulation of metals (Cd, Zn, Pb, and Cu) under the influence of EBl has been studied for different agricultural plants such as barley, tomatoes, radish, and sugar beet (Voronina *et al.*, 1997; Mineev *et al.*, 1996).

It was found that the application of EBl in appropriate doses in a certain stage of development reduces the metal absorption significantly. For example, the results obtained for barley plants (cv. Zazersky) treated with EBl by spraying in the booting stage at a dose of 10 mg/ha showed that the diminution of metal content in the plants was 40-98% in comparison with the control (Table XXIII).

TABLE XXIII

Effect of EBl on Heavy Metal Absorption in Barley Plants

Treatment	Content of metal, mg/kg			
	Cd	Pb	Zn	Cu
control	18.30	1.47	17.20	2.20
EBl	0.38	0.70	10.40	1.30

TABLE XXIV

Effect of EBl on the Uptake of Heavy Metals from the Soil into Tomato Fruits

Treatment	Content of Cd, mg/kg		Content of Zn, mg/kg	
	Fruits	Soil	Fruits	Soil
Me[a]	0.05	10.20	5.0	340
EBl[b]+Me	0.02	9.25	3.3	320
EBl+Me+EBl	0.04	4.28	3.6	280

[a] Addition of the corresponding salt to the soil. [b] Treatment with EBl.

Soaking tomato seeds (cv. Ranniy) for 12 h in a 10^{-8} M solution of EBl before sowing was more efficient in decreasing the content of Zn and Cd in tomato fruits than a double treatment consisting of soaking the seeds and spraying the plants in the budding stage with a 10^{-7} M solution of EBl (Table XXIV). However, in the last case, the atomic absorption spectroscopic analysis of the soil where the tomato plants were grown showed a lower content of the metals than in the case of the single treatment by soaking the seeds.

Sugar beet plants (cv. Bordo) were treated with EBl at a dose of 5 mg/ha in stage 3 true leaves. The content of Pb in beet roots was more than 50% lower and the content of Cd was 8% lower than in the control. It should be pointed out that the crop yield in this experiment was not higher than in the control. This means that this effect cannot be explained by dilution in the plant tissues but probably is connected with the regulation by EBl of the metal ion transport through the cell membranes.

Similar behavior concerning the accumulation of radionuclides under the influence of BS was found recently for different plant species. Experiments with barley plants (cv. Zazersky) on the absorption of cesium and strontium ions were carried out under laboratory conditions using modeled soil with added cesium and strontium salts. It was shown that changes in the ion content were influenced by EBl and depended on the stage of plant development. Table XXV illustrates the ion content in plants which were treated with 0.01 ppm of EBl solution in the

TABLE XXV

Effect of EBl on Cesium and Strontium Accumulation by Barley Plants

Treatment	Cs,Sr content in plants (2 weeks after treatment)			
	Cs		Sr	
	mg/g dry weight	% of control	mg/g dry weight	% of control
1. control	0.063	100	0.025	100
2. control + Cs - 27 mg/kg, Sr - 8.3 mg/kg	0.095	151	0.036	144
3. EBl, soil as in no. 2	0.068	108	0.030	120

booting stage and then were mowed after 2 weeks and dried (Khripach *et al.*, 1995g).

The Cs content in EBl-treated plants was close to that in the unpolluted control and was different from that in EBl-untreated plants by 43%. The Sr content was 24% lower than that in the polluted control. The dynamics of changes in ion accumulation during the vegetation period (Table XXVI) was quite favorable with respect to Cs and Sr in grains (70 and 36% lower than for the untreated plants, respectively) and was less evident for straw.

In much the same way, a minimization of ^{137}Cs accumulation after EBl treatment (0.01 ppm solution, spraying) was observed for Timothy plants (*Phleum pratense*) grown in an area with a high level of radioactivity (13.8 Ci/km^2). The radioactivity of treated plants was 27% lower than that of untreated plants grown under the same conditions: 27.0 and 37.0 Bq/kg, respectively.

TABLE XXVI

Effect of EBl on Cesium and Strontium Absorption by Barley Grain and Straw

Treatment	Cs,Sr content							
	Grain				Straw			
	Cs		Sr		Cs		Sr	
	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
1. control	0.016	100	0.003	100	0.069	100	0.062	100
2. control + Cs -27mg/kg, Sr - 8.3 mg/kg	0.038	238	0.008	236	0.181	262	0.082	132
3. EBl, soil as in no. 2	0.027	168	0.006	200	0.174	252	0.075	120

A comparison of the efficiency of two methods of EBl application to prevent ^{137}Cs accumulation in lupine plants (*L.luteus*, cv. Zhemchug) showed that the treatment of plants by spraying with a 10^{-9} M solution of EBl in the flowering stage gave better results than soaking seeds in a 10^{-9} M EBl solution (Zabolotny *et al.*, 1997). The data summarized in Table XXVII were obtained from an experimental area with a 72.5 Ci/km^2 level of radioactivity for ^{137}Cs. The first method of EBl application gave a significant diminution of ^{137}Cs content both in vegetative and in reproductive organs. When the seeds were treated, the content of ^{137}Cs in the plants at the flowering stage was even higher than in the untreated control. Fully maturated plants showed some decrease of ^{137}Cs content, especially in the vegetative organs, probably as a result of vegetative dilution.

TABLE XXVII

Effect of EBl Treatment on ^{137}Cs Content in Plants of *L. luteus* (cv. Zhemchug), Bq/kg of Dry Weight

Treatment	Flowering stage	Full maturity stage		
	Vegetative weight	Vegetative weight	Bean shells	Seeds
control	4038	3338	3687	4842
EBl, seeds	5956	2771	3634	4763
EBl, plants	-	2054	2528	3355

An application of EBl by treatment of the seeds as described above to the lupine plants (*L. angustifolius,* cv. Helena) and some cereal plants showed no effect on ^{137}Cs accumulation.

Additional confirmation of the ability of BS to regulate radionuclide absorption in plants can be concluded from the data obtained for *Calamagrostis epigeios* L. plants grown under natural conditions with a high level of radioactivity (59.9 Ci/km^2). The experimental plants were sprayed with a 10^{-9} M EBl solution either at the beginning of the growth or in the heading stage. γ-Radiometric analysis of the plants was first performed at the heading stage and then was done again at the end of the vegetation period.

Summarized data, including both plant and soil radioactivity, together with the transfer factors (TF) are shown in Table XXVIII. The TF is a ratio of radioactivity of the weight of dried plants in Bq/kg to the radioactivity of the weight of dried soil in Bq/kg, and it reflects the transfer of radioactivity from the soil to the plants.

The data in Table XXVIII show that EBl application caused a significant reduction of the transfer of radioactivity from soil to plants; the TF for treated plants was about 2.4-4 times lower than for untreated plants.

TABLE XXVIII

Effect of EBl Treatment on ^{137}Cs Accumulation in Plants of *Calamagrostis epigeios* L., Bq/kg of Dry Weight

Treatment	Sampling, dates and activity					
	3 June		TF	14 August		TF
	Plant	Soil		Plant	Soil	
control	3263	3126	1,04	3513	1710	2,05
EBl, 16 April (growth beginning)	3811	5626	0,68	2991	5626	0,53
EBl, 3 June (heading stage)	-	3754	-	3166	3754	0,84

An explanation of the observed BS-induced alterations in metal ion uptake from the environment to plants might be the influence of BS on the competition between ions in K^+-Cs^+ and Ca^{2+}-Sr^{2+} pairs for their uptake by the plant. This was checked under laboratory conditions using barley plants (cv. Roland). After growing as water culture, 7-day-old seedlings were put in a nutrient medium with equivalent amounts of the mentioned ions (Vedeneev *et al.*, 1997a). Half of the experimental plants were sprayed with an EBl solution (0.1 and 0.01 ppm), and the rest was treated by addition of EBl to the nutrient medium at the same concentration. EBl-untreated plants grown in the same nutrient medium were used as an experimental control in addition to a pure control without added Cs and Sr salts.

Five days of culturing in the presence of Cs^+ and Sr^{2+} led to the inhibition of seedling growth and development and to diminution of biomass. The seedlings that were treated with EBl grew better. The presence of EBl in the nutrient medium reduced the Cs^+ absorption by a factor 1.3 in comparison with the K^+ absorption, but the ratio of absorbed Ca^{2+} and Sr^{2+} was not changed. This effect

was found to be independent of the EBl concentration. For the seedlings sprayed with EBl, Cs^+ absorption was 1.6 times lower than K^+ absorption, and Sr^{2+} was absorbed 1.5 times slower than Ca^{2+}.

In the soil culture barley plants were treated in the tillering stage with an EBl solution (0.01 ppm). This had little effect on plant growth and development. Two weeks after the EBl treatment the enhancement of uptake of Cs^+ and Sr^{2+} by the plants was not significant, but for untreated plants it became 1.5 times higher. These data suggest the existence of a K^+-Ca^{2+}-dependent mechanism of regulation of radionuclide absorption by plants, which is influenced by BS.

D. EFFECT ON PROTEIN AND NUCLEIC ACID METABOLISM. STRESS RESPONSE

The obligatory precondition of growth, cell elongation and cell division, is an active biosynthesis of proteins. The increase in the rate of protein synthesis has to be preceded by activation of transcribing nuclear DNA into RNA, which is catalyzed by the DNA-dependent RNA polymerases. Increases in their activity ultimately will result in an increased number of ribosomes and in activation of protein synthesis. Indeed, the study of the nucleic acid-protein metabolism in plants treated with BS showed an activation of DNA and RNA polymerases and an increased level of DNA, RNA, and protein biosynthesis.

The first model plant systems, pinto bean and mung bean, were chosen because of their known high response to BS action and the ability to differentiate the BS-induced response from that of other plant hormones (Kalinich *et al.*, 1985, 1986). In pinto beans, excised swollen and split internodes were compared with the excised internodes of untreated controls. In mung beans, hypocotyls and epicotyls from Bl-treated and untreated seedlings were compared.

It was found that treatment with Bl resulted in increased RNA polymerase activity in both experimental models. As could be expected, in swollen tissue of

pinto beans the RNA polymerase activity was the greatest. The increase in enzyme activity led to a higher protein production, which directly linked to cell elongation. The RNA polymerase activity was also increased together with the protein production in split internodes but not to the same extent as in swollen tissue. It should be pointed out that these effects were strictly correlated with the data on elongation and weight changes of tissue sections obtained for the same plant systems.

In mung beans, Bl treatment resulted in cell division and elongation of the epicotyls while the morphology of hypocotyls remained relatively unaffected. An interesting feature of this system was that, nevertheless, the RNA polymerase activity was increased in both types of mung bean tissue, but to a greater extent in epicotyls. The parameters of protein synthesis followed the activities of RNA polymerases.

Similar alterations for both plant systems were found in the activity of DNA-dependent DNA polymerases after treatment with Bl. Because the enhanced activities of RNA and DNA polymerases could affect the nucleic acid level, RNA and DNA contents in the excised bean sections were also measured, and an enhancement was found in the tissues of all treated plants. A characteristic detail was the higher increase in DNA polymerase activity in split tissue than in the swollen tissue of pinto beans. This caused a greater increase in DNA content and a higher level of cell division. Thus, a close correlation between the morphological changes resulting from Bl treatment, on one side, and increased activities of the RNA and DNA polymerases and an enhanced level of proteins, RNA, and DNA, on the other side, was observed. Both enzyme systems were enhanced by Bl treatment, although one system was predominant in each type of tissue. The system that was enhanced most determined the tissue response. Further investigation (Mandava *et al.*, 1987) using selective inhibitors of RNA and protein synthesis on EBl- and (22*S*,23*S*)-EBl-induced responses in mung

bean confirmed the previous results and showed that the decline in epicotyl growth caused by the inhibitors can be overcome by the effect of BS.

These results led to the assumption that Bl acted on the genome in the form of a hormone-receptor complex. The action of Bl resulted in genome activity regulation and could be responsible for the effects of Bl on transcription and DNA-replication. This hypothesis was examined further to reveal the main sites of BS action inside the cell and to learn the details of its molecular mechanism. If the hypothesis is realistic, specific genes that are transcriptionally regulated by BS must exist. Thus, investigations of the effect of BS on gene expression, mostly *via* examination of a variety of specific gene products, became one of the directions in studies of the mechanism of action of BS. The studies on the effect of Bl on gene expression using a molecular-genetic approach (Clouse *et al.*, 1992; Zurek and Clouse, 1994; Zurek *et al.*, 1994) led to characterization of the BS-regulated gene from soybean (Zurek and Clouse, 1994). Although, the problems formulated initially when this program was started (Clouse and Zurek, 1991) are not fully solved, this approach did bring progress in the understanding of the mechanism of action of BS. The main difficulties for further developments are caused by the extreme multifunctionality of BS in plants, which is not connected with the genetic level only.

An attempt to localize a starting point in the chain of BS-initiated signal transduction was undertaken in studies on the action of BS on RNA and protein biosynthesis in genetically different barley (Deeva *et al.*, 1996b) and wheat plants (Mazets and Deeva, 1996; Deeva *et al.*, 1997a; Mazets, 1997).

To distinguish the effects of the nuclear and the cytoplasmic genome, two varieties of barley plants, Roland (R) and Zazersky (Z), and their isoplasmatic lines R(Z), bearing the nucleus of the Roland variety and the cytoplasm of the Zazersky variety, and Z(R), bearing the nucleus of the Zazersky variety and the cytoplasm of the Roland variety, were chosen as experimental plant systems to study the effect of EBl application on the qualitative and quantitative

composition of RNA and of soluble and structural proteins. The Roland variety is characterized by a higher rate of nucleic acid and protein biosynthesis, by a higher heterogeneity of the polypeptide composition of soluble proteins in organelles, and by an increased metabolic activity of some enzymes, e.g., alanine and aspartate-aminotransferases. Such differences are very important for the plant response to external influences.

Two variants of EBl application were used: (1) soaking the seeds in a 0.01 ppm EBl solution and (2) spraying the plants with the same solution in the tillering stage. It was found that EBl did not affect the qualitative composition of the RNA fraction but changed the content of some its components. In the Zazersky variety EBl enhanced the quantity of tRNA and rRNA, and in the isoplasmatic line Z(R) their synthesis was slower, especially that of tRNA. In the Roland variety EBl enhanced the content of tRNA more efficiently than in the Zazersky variety. In the line R(Z) the synthesis of (28*S*)- and (18*S*)-rRNA was also increased.

Qualitative and quantitative changes were found in the composition of soluble and insoluble proteins in the EBl-treated plants. The character of these changes was dependent on the genotype and on the time of observation. In the case of the Roland variety and in the line R(Z), new polypeptides in the region of 67 and 30-20 kDa appeared in the electrophoretic spectra of soluble proteins, 5 h after the plants were sprayed with EBl. In the line R(Z), some components in the high-molecular-mass region were decreased additionally. After 72 h in the Roland variety, treated plants showed only changes in the low-mobility components, and in the R(Z) line, in some components with low and middle mobility. The changes in the high-molecular-mass region in both genotypes were similar after treatment with EBl. Polypeptides with REM (relative electrophoretic mobility) 0.13 and 0.17 were induced and two components with MW 80 and 43 kDa were degraded. An interesting detail of the study (Deeva *et al.*, 1997b) was the comparison of the EBl effect with the changes in the protein

spectrum induced by the nonnatural synthetic plant growth regulator "kvartazin" [*N*,*N*-dimethyl-*N*-(2-chloroethyl)hydrazine chloride]. Under the same conditions it led also to a change of intensity of some components in the protein spectrum, but not to the appearance of new components.

In the Zazersky variety a new polypeptide with REM 0.40 was found only 48 h after the application of EBl. The intensity of the component with a MW of 43 kDa was decreased, and the component with a MW of 30-20 kDa was increased. Similar changes in the electrophoretic spectra of soluble proteins were observed in the line Z(R); however, *de novo* synthesis of the corresponding polypeptides had not occurred.

The most significant changes in the composition of soluble proteins in the Zazersky variety were found 72 h after the application of EBl. The intensity of the already existing components with REM 0.31 and 0.34 became higher for the first one and lower for the second one. Besides, the components with REM of 0.22 and 0.24 disappeared, and a new polypeptide with REM of 0.26 was found. The synthesis of polypeptides with medium mobility was activated, and a new highly mobile component (REM 0.92) was induced (Fig. 43). The changes in the line Z(R) after the application of EBl mostly followed the behavior of the nuclear donor (Fig. 44).

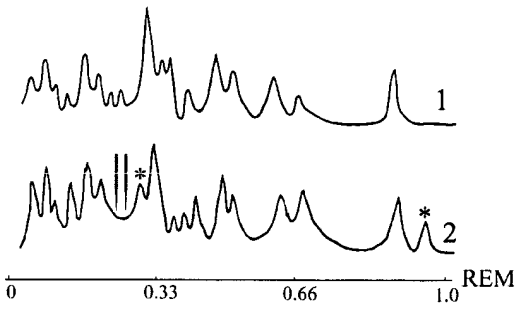

Fig. 43. Densitogram of the soluble proteins in barley plants cv. Zazersky: 1 - control, 2 - treatment with EBl; * - induction, ↓ - degradation.

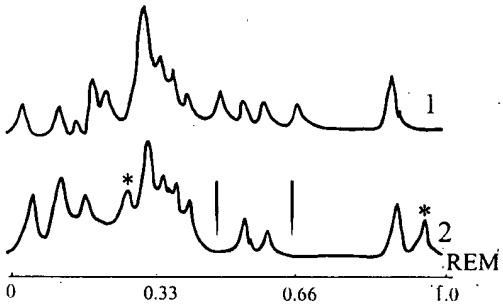

Fig. 44. Densitogram of the soluble proteins in barley plants cv. Zazersky (Roland): 1 - control, 2 - treatment with EBl; * - induction, ↓ - degradation.

The treatment with EBl had a different effect on the composition of structural proteins in the different genotypes and it came more to expression 72 h after treatment. The Roland variety and the line R(Z), bearing its nucleus, showed similar changes in protein content: a relative diminution of the high-molecular-mass polypeptides and an increase of the middle- and low-molecular-mass polypeptide components. In both cases a new peptide with a REM of 0.90 was found. A similar tendency in changes of the protein spectrum under the influence of EBl was found also for the Zazersky variety and for the line Z(R) bearing its nucleus.

A conclusion that can be drawn from the obtained results is that the effect of BS on the protein biosynthesis is mainly controlled by the nuclear genome, although the role of cytoplasmic structures cannot be excluded.

In another study, based on the employment of experimental plant systems with fixed genetic differences, the effect of EBl on the protein biosynthesis in wheat cv. Chinese Spring was investigated. The euploid and two related DT-lines, which differed from the euploid by the absence of a short ($5B^L$) or a long ($5B^S$) arm in their chromosomes, were compared with respect to their reaction to a treatment with EBl (Mazets and Deeva, 1996; Mazets, 1997; Deeva *et al.*, 1997a). A specific feature of the approach was the parallel analysis of shifts in growth, hormonal spectrum, and protein synthesis. The mentioned lines were

chosen out of 18 initially studied DT-lines because of their characteristic BS-dependent behavior.

Six-day-old plants were treated with a 10^{-7}% solution of EBl by spraying. The absence of one of the chromosome arms led to changes in plant growth rate, reduction of phytohormone accumulation, and deviations in protein synthesis resulting in changes in the quantitative and qualitative composition of soluble proteins. The effect of EBl in treated plants was visible even in the early stages of plant growth. EBl reduced the shoot weight of the euploid and to a lesser degree that of the DT-line $5B^L$ 3 days after treatment. The shoot weight was slightly increased at the end of the experiment (Fig. 45). The shoot weight of the line $5B^S$ was increased in the beginning, but lowered till 120 h after the treatment. The length of shoots was changed to a lesser extent in the same period.

The changes in shoot growth were accompanied by differences in accumulation of IAA. The quantity of IAA was noticeably decreased shortly

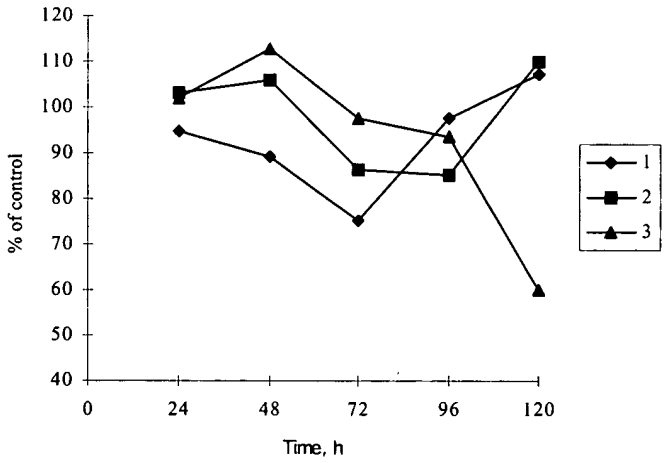

Fig. 45. Effect of EBl on shoot weight of euploid and DT-lines of wheat cv. Chinese Spring (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

after EBl treatment of the euploid, but later it increased and stabilized after 96 h (Fig. 46).

The IAA content in the $5B^L$-line showed an enhancement in two steps and was closer to that in the euploid than in the $5B^S$-line. The last one demonstrated an extremely high level of IAA in the beginning of the experiment and a dramatic reduction of this content in the final period. This tendency of higher initial EBl activation of the plants with more deficiency in genetic material was found also in the shoot development, as shown above, and in the EBl-induced regulation of the soluble protein synthesis (Fig. 47). Such a correlation between the effect of EBl and the genome looks like a compensatory action of EBl, which could take place when EBl-regulated genes are not present in the lacking part of the chromosome.

The quantitative and qualitative compositions of RNA and proteins were affected by EBl, and the *in vivo* synthesis of soluble proteins was activated in the euploid and in the lines, except for the $5B^L$-line on the first day after the treatment.

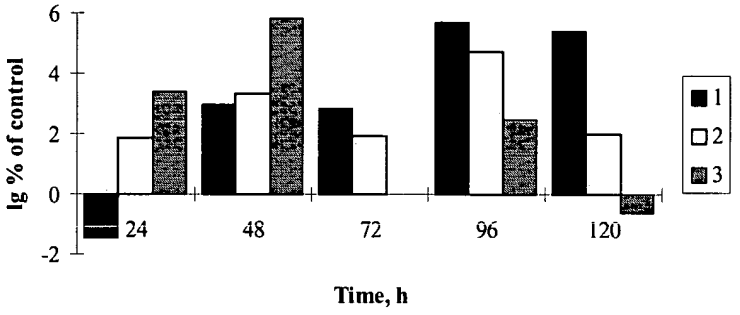

Fig. 46. Effect of EBl on the content of free IAA in the euploid and in the DT-lines of wheat cv. Chinese Spring (1 - euploid, 2 - $5B^L$-line, 3 - $5B^S$-line).

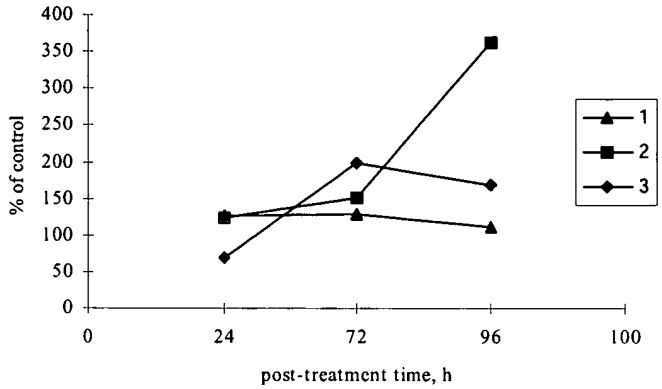

Fig. 47. Effect of EBl on the synthesis of soluble proteins in the euploid and the DT-lines of wheat cv. Chinese Spring (1 - euploid, 2 - $5B^S$-line, 3 - $5B^L$-line).

Many data published in the literature during the period of BS investigations indicate, directly or indirectly, a strong influence of BS on the protein metabolism. Such data were obtained for wheat and mustard plants (Braun and Wild, 1984a), celery plants (Wang, Y. *et al.*, 1988), rice (Mai *et al.*, 1989), radish (Beinhauer *et al.*, 1990), sugar beet (Schilling *et al.*, 1991), and some other crops. As a rule, this influence activates the protein synthesis and enhances the total protein yield. As discussed above, the changes in protein biosynthesis can be observed at different levels, in the separate components of the protein spectrum, in the ratio of different components, and in the total protein yield. The last effect is well documented, for example for lupine plants that showed a positive effect of BS on the protein production in all experiments (Mironenko *et al.*, 1996, 1997; Kandelinskaya and Khripach, 1990). The results obtained in field experiments for two lupine varieties, *L. luteus* cv. BSKA and *L. angustifolius* cv. Omega, after spraying with two concentrations of EBl in the beginning of flowering are shown in Table XXIX. Although the changes in protein content for two varieties were quite different, a positive trend in protein accumulation by the seeds was observed.

TABLE XXIX

Effect of EBl on Protein Content in Seeds of *L. luteus* and *L. angustifolius*

Treatment	Protein content, % of dry mass	
	L. luteus	*L. angustifolius*
control	40.26	37.72
EBl, 10^{-9} M	43.13	40.22
EBl, 10^{-7} M	42.00	40.13

The treatment of seeds with Bl was less efficient with respect to the total protein accumulation, but it gave an opportunity to study the Bl-influenced protein synthesis in the early stages of plant development and to estimate the range of active concentrations (Fig. 48).

Together with quantitative shifts in total protein, some qualitative changes were also registered. It was shown, for example, that the legumine fraction of the total proteins of the seeds obtained from treated plants was about 1.5 times higher than in the control. In addition, the study of the activity of some enzymes

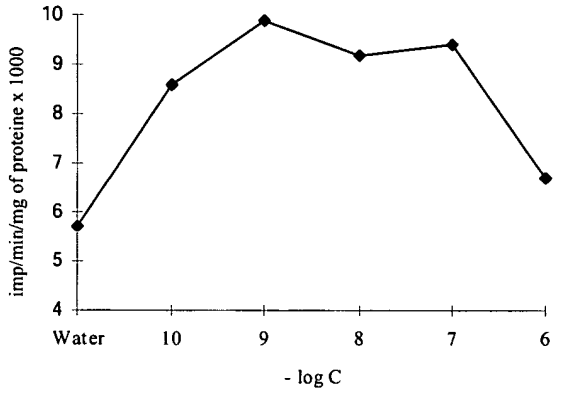

Fig. 48. Effect of Bl on the rate of protein synthesis in embryos of *L. luteus* after treatment of seeds with different concentrations of Bl (rate of incorporation of [^{14}C]leucine).

involved in the regulation of the protein metabolism showed that Bl and EBl did not affect the neutral protease activity.

A comparison of the behavior of growing seedlings and ripening seeds showed that both Bl and EBl act as anabolics in relation to chlorophyll, DNA, RNA, and protein synthesis in cotyledons and embryos in growing seeds. The character of action of the hormones was similar, but in heat stress conditions EBl was more efficient. Similar effects took place also in maturated seeds. The total nucleic acid content was increased in treated plants till the 26th day after EBl application, and it mainly resulted from enhancement of the DNA content. In this period the activity of hydrolytic enzymes, DNAases, and RNAases was decreased and started to regenerate gradually till the 37th day after treatment (Fig. 49).

The obtained results could be interpreted as an anabolic effect of BS in the growing plant and in the maturating seeds created by initiation of synthetic processes, by partial inhibition of catabolic processes, and by preservation in this way of tissue functions as centers of assimilate attraction.

An interesting aspect of the influence of BS on protein metabolism is the change found in the amino acid composition of the total protein. The data indicate significant shifts in the content of some amino acids in the total protein

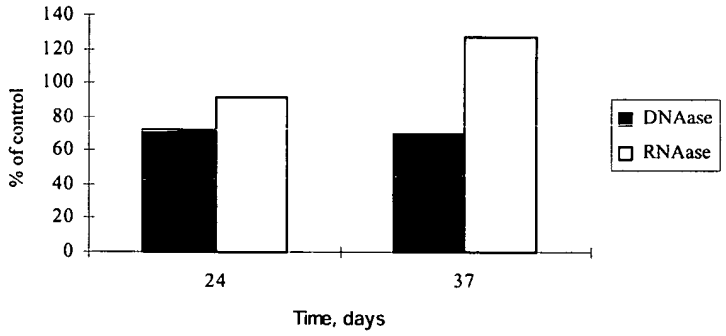

Fig. 49. Nuclease activity in ripening seeds of *L.luteus* after treatment with EBl (10^{-9} M/l).

TABLE XXX

Content of Some Amino Acids in the Total Protein Content of Seeds of *L. luteus*, %

Amino acid	Treatment	
	Control	EBl, 10^{-9} M
methionine	0.35	0.51
lysine	4.87	5.32
cysteine	3.14	4.35
glutamic acid	22.92	21.50
tyrosine	2.72	3.03

content of lupine seeds obtained from plants treated with EBl (Table XXX) (Mironenko *et al.*, 1997).

These data are important not just to indicate how the protein nutrient value is affected by BS but also as a possible characteristic for the plant stress resistance, because some amino acids are known to be important for plant stress response. Along with the amino acids, osmoregulating metabolites such as choline, betaine, and other tertiary ammonium compounds (TAC) are interesting in this respect. The balance of these substances in the cell regulates to a large extent the physicochemical status of the cell matrix and affects the mesomorphic membrane structure *via* quick alterations in the content of some components. Accumulation of these substances by plants increases their adaptive ability to external factors. Research on the effect of EBl on the accumulation of these compounds by barley plants was recently carried out (Deeva *et al.*, 1997b). The data shown in Fig. 50 were obtained in the tillering phase for barley plants (cv. Roland) grown from seeds treated with a 0.01 ppm EBl solution. Similar results were observed for the same plants when sprayed in the tillering phase.

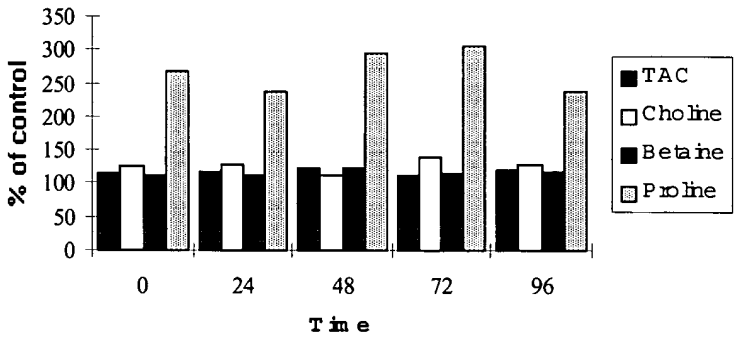

Fig. 50. Effect of EBl on the content of osmoregulating metabolites in barley plants.

The accumulation of all metabolites was activated in EBl-treated plants and was quite high during the studied period. The change of the proline content was most significant. It was about 3 times higher than the control in the period between 48 and 72 h after the beginning of the measuring.

A specific feature of this research was a simultaneous study of similar effects of some other nonsteroidal synthetic plant growth regulators under the same conditions applied either separately or as a mixture with EBl. It was found that in some cases the effect of these regulators was rather high and additive with EBl. After a short period that was comparable with the duration of their metabolic degradation, the total effect of the mixed treatment was decreased to the level of EBl treatment alone, which was quite stable and continuous.

These and other experiments gave additional information on the nature of the adaptogenic effect of BS, which may be connected with selective changes in specific links in metabolic pathways initiated *via* gene expression and synthesis of certain enzymes, proteins, and accompanying metabolites. An essential contribution to the understanding of the mechanism of the antistress activity of BS was made by investigations on BS-influenced protein synthesis in wheat leaves at normal and high temperature (Kulaeva *et al.*, 1989). High temperature was used as a stress factor causing heat shock protein synthesis, which is

considered to be important in effecting of a protective response in plants. It was found that both EBl and (22*S*,23*S*)-HBl activated in stress conditions and in normal conditions the total protein synthesis and initiated essential shifts in the protein spectrum. An interesting feature was that EBl and (22*S*,23*S*)-HBl induced protein patterns similar to heat shock protein patterns not only under stress but also at normal temperature. This resulted in enhancement of the thermotolerance of protein synthesis in BS-treated plants, an effect that is connected with an increase of the thermostability of membranes.

The protection of cucumber plants against heat stress after treatment with BS was observed at high (40 °C) and at low (5 °C) temperature and it resulted in a significant increase of the germination ability of treated seeds, better growth, higher dry mass, and more chlorophyll accumulation (Katsumi, 1991). Similar data on the promotion of the chilling resistance of maize seedlings by Bl resulting in better growth recovery after rewarming were reported (He *et al.*, 1991). Bl also increased the ripening of rice plants under low-temperature conditions (Hirai *et al.*, 1991).

A comparison of the nature of the temperature stress protective action of BS with similar properties of ABA in bromegrass (*Bromus inermis*) as a plant model system showed different mechanisms by which these compounds exert antistress effects (Wilen *et al.*, 1995).

A protective effect of BS in salt stress conditions was shown for barley plants grown from seeds treated with (22*S*,23*S*)-HBl or EBl (Kulaeva *et al.*, 1991; Bokebaeva and Khripach, 1993). Electron microscopy showed that treatment with BS prevented the degradation of the cell ultrastructure, which was induced by a 0.5 M solution of NaCl. Some changes in ion content and an enhancement in chlorophyll accumulation were found in 7-day-old plants.

A decrease of the effect of water stress in mung beans treated with EBl was shown to be connected with a higher ability of plants to assimilate water, which was confirmed by experiments with tritiated water (Zhao and Wu, 1990). At the

same time, a significant increase of proline content was found in treated plants, which was interpreted by the authors as an indication for the increase of the resistance of the plants to stress conditions.

Application of (22*S*,23*S*)-HBl in rather high doses (1 g/ha) to sugar beet plants in conditions of mild water stress did not lead to a stress reaction while in the control a loss of 8% of biomass was observed (Schilling *et al.*, 1991). Treatment with BS activated the growth of the lateral roots by 25-30% in comparison with untreated plants under stress conditions.

E. RESISTANCE TO DISEASES

Although the stress-protective properties of BS have been known for a long time, systematic investigations of the potential of BS to enhance plant resistance to diseases, which can be considered also as a kind of stress, were started only recently. Among the results obtained to date in this area, the largest part is connected with the influence of BS on fungal phytopathogenesis. The early data obtained for potato plants as a model system showed that treatment of plants or sowing material with BS could significantly affect the development of fungal infection. The treatment of plants was found to be protective against fungal infection for all the applied doses of BS, but the treatment of tubers sometimes activated the disease. Thus, investigation on the interaction between *Phytophthora infestans* (Mont.) de Bary and potato showed that EBl and HBl induced a higher susceptibility of potato tuber tissues in the concentration range of 10^{-8}-10^{-16} M (Vasyukova *et al.*, 1993, 1994). This effect was caused by stimulation of the growth of mycelia of the fungi and enhancement of spore formation. This was confirmed by a direct experiment where fungi were grown in media that contained EBl in different concentrations. The highest concentration, 10^{-5} M, induced lysis of zoospores. Lower concentrations did not affect the growth of fungi, but starting from a concentration of 10^{-10} M, the

stimulation of hyphae growth increased to its maximum at 10^{-14} M and then it decreased gradually, although even at 10^{-20} M some stimulation could be seen. A specific feature of the EBl action was the influence on the vegetative growth of fungi, but not on the reproductive functions. The last mode of action is typical for some phytosterols that activate the process of spore formation in sterol-depending fungi. An additional explanation for the observed phenomenon of pathogen initiation as a result of weakening of the immune status of plant tissues was suggested based on data on the inhibition of wound tissue reparation under the action of BS in the absence of phytopathogen.

The data (Korableva *et al.*, 1991, 1992, 1995; Platonova *et al.*, 1993) suggest that the mentioned results can be considered from different points of view. It was shown that treatment of potato plants or tubers after harvesting led to a prolongation of the period of deep dormancy of tubers and to enhancement of their resistance to phytophthora infection and other diseases. Under the influence of EBl the production of ethylene by tuber tissues increased and the biosynthesis of protective substances of phenolic and terpenoid nature was activated. These effects took place when the intact tubers were treated with EBl at a concentration of 0.1-0.01 mg/l, which was higher than the stimulative concentration for pathogen development. This means that the protective or deprotective type of activity of BS depended on the method and time of BS application and was connected with the different stimulating points of either the plant or the pathogen. In such cases the desirable result can be achieved by choosing the optimal conditions.

As mentioned above, the treatment of potato plants with BS essentially decreased the level of phytophthora development. It was shown in field experiments that most efficient in this respect was spraying plants with BS (Bl, EBl, and HBl) solutions in doses of about 10-20 mg/ha in the beginning of the budding stage (Khripach *et al.*, 1996c, 1997) (Table XXXI).

TABLE XXXI

Effect of BS on Potato Plant Resistance to Phytophthora Infection

Treatment	Tubers affected by disease		
	number/1000 tubers	% of total amount	% of control
cv. Orbita			
control	19	1.9	100
HBl	16	1.6	84
EBl	17	1.7	89
cv. Sante			
control	25	2.5	100
EBl	19	1.9	76
cv. Rosinka			
control	29	2.9	100
EBl	19	1.9	66

In some cases the efficiency of BS in protection against fungi was even higher than for plants treated with standard fungicides. Usually, in conditions with a higher level of pest development, a higher expression of protective properties of BS was observed. The data shown in Fig. 51 were obtained for

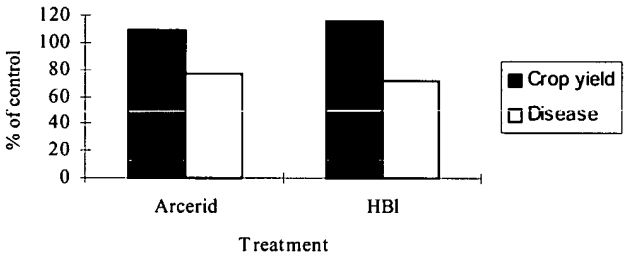

Fig. 51. Effect of HBl on the yield of potato tubers and resistance of plants to phytophthora infection in comparison with the standard fungicide arcerid (cv. Adretta).

potato plants treated with HBl at a dose of 20 mg/ha in the beginning of the budding stage. The protective effect against fungi produced by this single treatment with BS was similar to the effect produced by a double treatment with the standard fungicide arcerid (composition of ridomil and polycarbacine) at a dose of 2 kg/ha.

Later, a study on the mechanism of the protective effect of BS was carried out with barley plants as a model system in field and laboratory conditions. It was found that spraying plants in the tillering phase with a solution of EBl significantly decreased the extent of leaf diseases induced by *Helmintosporium teres* Sacc. under laboratory conditions. The effect of EBl on the resistance of barley plants to leaf diseases induced by mixed fungi infection in field conditions is illustrated by Fig. 52; the development of disease is indicated for the heading stage. This effect was accompanied by an increase in grain yield that was significant even at a dose of 5 mg of EBl per hectare (Pshenichnaya *et al.*, 1997; Volynets *et al.*, 1997a,b).

The highest level of disease suppression took place at a dose of 15 mg of EBl per hectare and it was comparable with the effect induced by the standard fungicide Bayleton when applied in the usual dose. An attractive feature of the

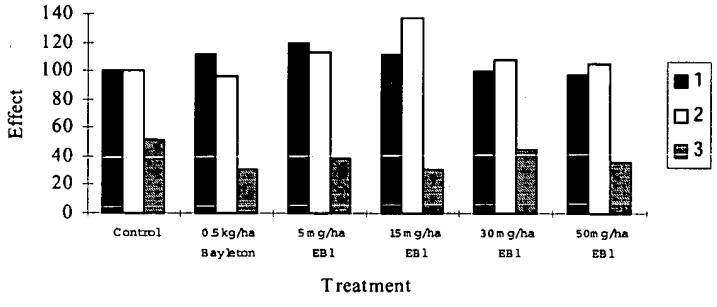

Fig. 52. Effect of EBl on barley plant resistance to leaf diseases and on productivity (cv. Prima Belarusi) (1 - bushiness, % of control; 2 - weight of grains, % of control; 3 - development of disease, %).

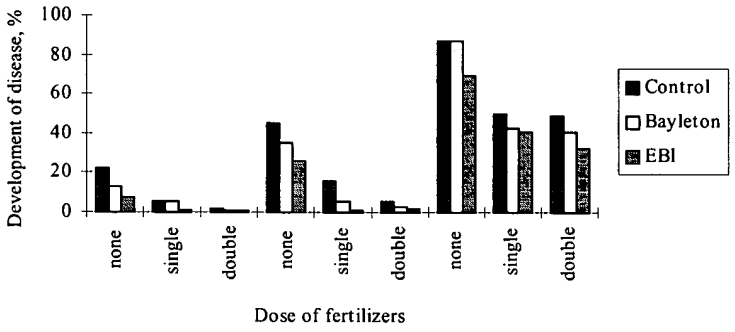

Fig. 53. Effect of EBI on barley leaf diseases at different doses of mineral fertilizers (cv. Prima Belarusi).

action of EBI was a simultaneous stimulation of the plant fungi protective properties and the higher productivity that was observed for all doses.

An interesting result on BS-regulated resistance of barley plants to leaf diseases was obtained during the study of the effect of EBI at different doses of mineral fertilizers. All experiments showed a protective effect of EBI, which was especially significant at a high dose of fertilizers (Fig. 53).

The data shown in Fig. 53 were obtained for a 5 mg/ha dose of EBI and a usual (0.5 kg/ha) dose of Bayleton. They illustrate the higher capacity of EBI as a protective factor against fungi under these conditions in comparison with the traditional fungicide.

In separate experiments (Pshenichnaya *et al.*, 1997b) it was shown that the fungistatic activity of EBI, measured in cultures of the fungus *H. teres,* was rather low (the active concentration was higher than 20 mg/l). So, it was concluded that only a hormonal effect, leading to activation of the internal mechanism of plant resistance, could be responsible for the observed results. Since regulation by a hormonal signal has to include a series of biochemical shifts, some metabolic parameters of plants infected with *H. teres* Sacc. after treatment with BS were investigated (Volynets *et al.*, 1997a).

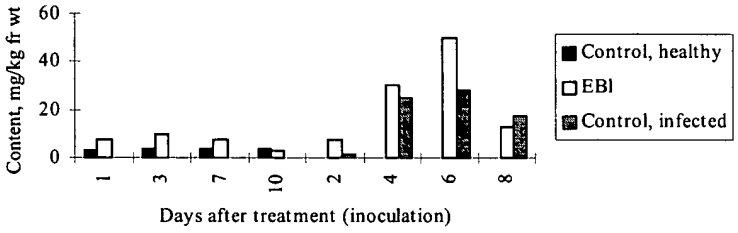

Fig. 54. Content of auxin in leaves of barley (cv. Zazersky) infected with *H. teres* Sacc.

The treatment of barley plants with EBl promoted the accumulation of auxin, and the same tendency was observed with the treatment of infected barley plants (Fig. 54).

It has been shown that the main reason for changes in the auxin level was the inhibition of auxin-oxidase activity under the influence of EBl in the infected barley plants (Fig. 55).

Simultaneously, the plants treated with EBl showed a higher peroxidase activity and an increased level of some phenolic components in comparison with the control, for both the healthy and the infected variant of the experiment. The total activity of gibberellins was not influenced under these conditions.

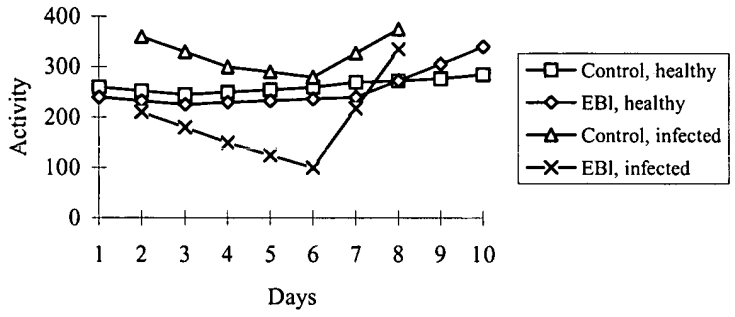

Fig. 55. Activity of IAA oxidase in leaves of barley plants (cv. Zazersky) infected by *H. teres* Sacc. and treated with EBl.

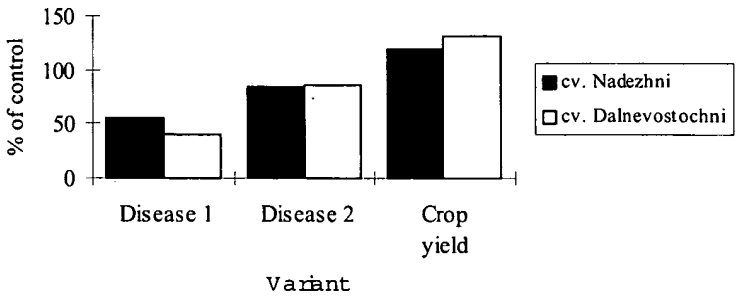

Fig. 56. Effect of EBl on the yield of cucumbers and the resistance of the plants to peronosporosis (disease 1 - middle of vegetation period; disease 2 - the end of vegetation perion).

A protective effect of EBl against fungi was established in field trials using cucumber plants as a model system (Churikova and Vladimirova, 1997). Figure 56 illustrates the results on suppression of peronosporosis in cucumber plants under the influence of EBl. In these experiments EBl was applied twice. First, seeds were soaked in a 0.1 mg/l solution of EBl and then the plants were sprayed at a dose of 25 mg/ha in the flowering stage.

An increase in the activity of some enzymes (peroxidase, polyphenoloxidase) in the leaves of cucumber plants has been found also. Since these enzymes are involved in the metabolism of polyphenols, a change in their activity may be considered as one of the factors that are connected with the increase of plant resistance to infection.

A new recently discovered aspect of the protective action of BS on plants is related to their ability to stimulate resistance to virus infection (Bobrick, 1993, 1995; Rodkin *et al.*, 1997). Potato starting material, produced from cuttings, was cultured in a medium that contained Bl, EBl, or HBl. This resulted in a significant increase of all the parameters that characterized the growth and development of the cuttings in comparison with the control and led to a higher yield of plants suitable for propagation by cutting. One of the most important

effects was found in the reduction of virus infection in the resulting starting material used for planting. This effect was found in all stages of plant development and was observed also in the first and in the second tuber generations produced from the starting plant material grown in BS-containing media. Figure 57 shows such a long-term EBl effect on the resistance to virus infection and productivity of potato plants grown from the tubers of the first tuber generation. Along with significant lowering of the virus infection, the plants obtained from BS-influenced sowing material gave a higher crop yield, which differed from the control to a maximum of 56%. The highest efficiency in crop increase corresponded to the lowest level of disease development. This was the case for plants for which the previous generation was produced from starting material that was grown in a nutrient medium with EBl at a concentration of 0.25 mg/l.

The presented data indicate that exogenous BS can act efficiently in plants as immunomodulators when applied in the appropriate dose and in the correct stage of plant development. As in other cases of BS-regulated stress response, the protective action of BS against pathogens is the result of a complex sequence of biochemical shifts such as activation or suppression of key enzymatic reactions, induction of protein synthesis, and stress substances of different nature. These

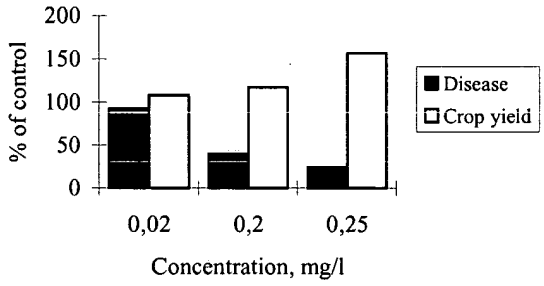

Fig. 57. Effect of EBl on the resistance to virus infection and productivity of plants grown from the first tuber generation (cv. Temp).

relatively little investigated properties of BS are very promising from a practical point of view. They open up new approaches for plant protection based on the employment of very small amounts of environmentally friendly natural substances instead of the traditional pesticides, which often conflict with the environment.

F. EFFECT ON THE PHOTOSYNTHETIC APPARATUS

Plant ontogenesis depends on two integral processes, which are closely related to each other. These processes are growth and photosynthesis. Existing theories describe the growth-photosynthesis relationship from different points of view. One assumption is that the expression of genes responsible for growth is independent from genes that code the biosynthesis of the photosynthetic pigments. The interconnection of these processes during further plant development is realized *via* the regulatory systems of the plant, particularly *via* a light-dependent growth regulating system. Together with its role as a source of energy for photosynthesis, light is a source of information for plant photomorphogenesis. It is assumed that the transduction of a light signal from the photoreceptors can be mediated by alterations in the endogenous phytohormone balance. It is known, for example, that the content of some phytohormones in tissues of plants grown in the dark can be changed very quickly after a short irradiation with red light and that this effect is mediated by phytochrome.

A possible role of brassinosteroids in this process was a subject of interest from the beginning of research on BS. A characteristic feature of BS action, their ability to increase a crop yield, suggested such a role because the improvement of photosynthetic efficiency is an important prerequisite for an increase in productivity. Indeed, the importance of illumination with light of the correct spectral quality was shown for BS-activated growth of beans, which correlated

with the accumulation of chlorophyll and photosynthetic assimilates (Krizek and Worley, 1973, 1981; Krizek and Mandava, 1983a,b; Kamuro and Inada, 1991; Kamuro and Takatsuto, 1991; Hirai *et al.*, 1985). Results on the enhancement of the photosynthetic capacity and the translocation of photosynthetic products after the application of BS were obtained in rice (Fujii *et al.*, 1991), in maize (He *et al.*, 1991), in wheat and mustard (Braun and Wild, 1984a,b), and in lupine plants (Mironenko *et al*, 1996). In the last case the stimulation of chlorophyll accumulation after application of Bl under normal conditions was accompanied by an increase of the thermoresistance of the photosynthesis under heat shock. Similar results with respect to chilling stress were obtained during investigations of the action of Bl on the growth of cucumber hypocotyls (Katsumi, 1991).

The data derived from these studies confirm that the action of BS is a light-dependent process. Some of these data are important for practical application of BS, showing for instance that the result of treatment is influenced by the photoperiod and can be different for long-day plants and short-day plants. Highly important from a mechanistic point of view were the data that illustrated a compensatory effect of BS in plant growth inhibited by specific light

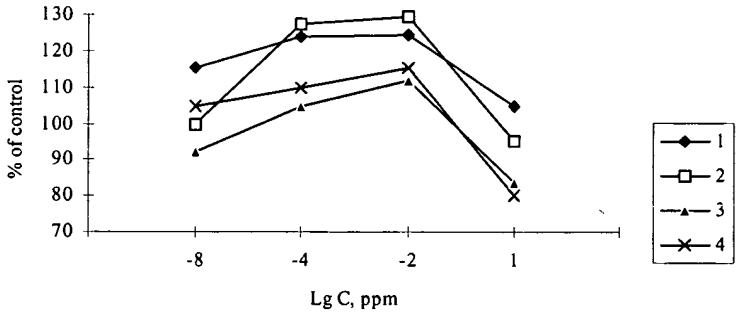

Fig. 58. Effect of different EBl concentrations on the chlorophyll accumulation in triticale seedlings: 1 - content related to area unit, leaf; 2 - content related to dry weight, leaf; 3 - content related to area unit, coleoptile; 4 - content related to dry weight, coleoptile.

conditions. This leads to the supposition of a relation between BS-induced growth and the action of phytochrome. To clarify this relationship some further experiments were carried out.

An investigation (Kalituho *et al.*, 1996) of the action of EBl on the formation of the pigment apparatus in young triticale plants in a period when the leaf changes its functions from a sink to a source showed that soaking the seeds with EBl stimulated the accumulation of chlorophyll in 6-day-old first leaf and in the coleoptile over a wide range of concentrations (Fig. 58).

The analysis of green seedlings showed that the maximal effect of EBl in the enhancement of the chlorophyll content took place in the earliest stages of plant development and in the period of leaf emergence from the coleoptile (Fig. 59).

The period when the leaf came out from the coleoptile was found to be the most sensitive to the action of EBl also in postetiolated plants. At first, the chlorophyll content in these plants was lower than in the control. This last effect could be considered as a confirmation of the role of light in the realization of the stimulative influence of BS on the pigment apparatus and as an indication for their probable participation in mediation of the phytochrome regulatory functions.

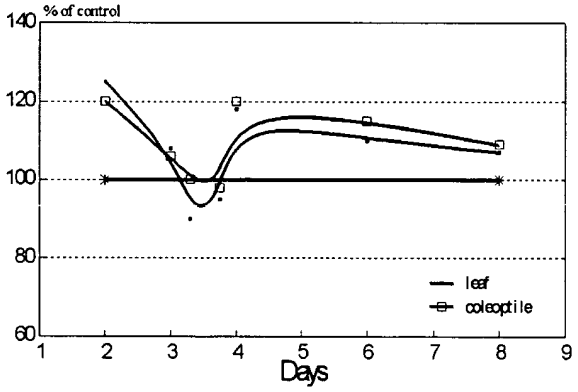

Fig. 59. Effect of EBl on the chlorophyll accumulation in leaves and coleoptiles of triticale seedlings grown under normal light conditions.

Further studies of the same plant model system under various light conditions, such as irradiation by white light, low-intensity flashing red (670 nm) light, far-red (730 nm) light, a combination of these, and dark, brought new support for this hypothesis (Kalituho, 1997; Kalituho *et al.*, 1997a). It was found that a short treatment of triticale seedlings with red or with a combination red + far-red light stimulated the chlorophyll and protochlorophyllide biosynthesis in the dark, while treatment with far-red light did not provoke chlorophyll synthesis in the dark and did not change the protochlorophyllide content significantly. During dark incubation the content of pigments increased till about 28 h of incubation, and after that the total pigment amount decreased. When the effects of successive irradiation by red and far-red light and those of only a red-light irradiation on pigment synthesis were compared, EBl-treated plants and untreated controls reacted differently. In red-light-irradiated plants the relative efficiency of pigment synthesis was higher in the EBl-untreated control; the red + far-red-irradiated plants showed a higher efficiency of pigment synthesis in the EBl-treated plants. This result probably means that the phytochrome-deactivating effect of far-red light was counterbalanced partially by the action of EBl, which could imply a phytochrome dependence of the action of EBl on the accumulation of photosynthetic pigments.

Further confirmation of this supposition was obtained from experiments with preilluminated plants which were exposed after 12 h of darkness to continuous white light. It was found that red light increased the rate of chlorophyll accumulation and that the combination of red + far-red light gave a lower effect in postetiolated triticale leaf. The effect of EBl on chlorophyll accumulation in red-light-pretreated plants was most significant in the early stages of greening.

Figure 60 illustrates the effect of red light and of EBl treatment, acting together or separately, on the chlorophyll accumulation in the first triticale leaf. The data were obtained for seedlings, which were postetiolated for 1 h. It was shown that the combined effect produced by light activation of phytochrome and

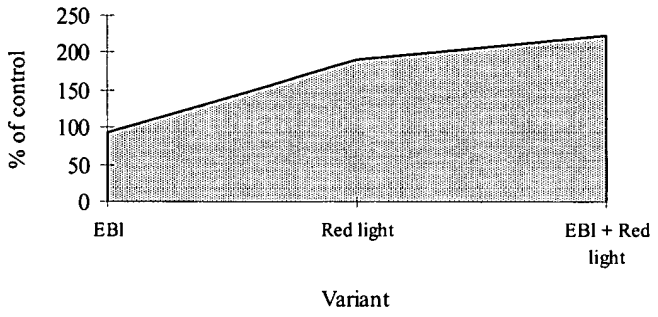

Fig. 60. Effect of red light and EBl treatment on the chlorophyll content in the first triticale leaf.

the action of EBl was higher than the effects of both factors separately; the effect of EBl alone was close to the control. These results suggest an essential role of phytochrome in the realization of the effect of EBl.

The mechanism of interaction of BS and the phytochrome system is unclear. It is possible that phytochrome$_{FR}$ formation under the flash of red light causes an alteration in the membrane permeability that could facilitate phytohormone transport or adhesion of BS by specific receptors. On the other hand, an interaction of BS and phytochrome at the genetic level cannot be excluded also, and their relationships in different plant systems should be investigated further.

A new intriguing aspect of the relationship between BS-growth-regulating and light-growth-regulating effects in plants is connected with the recent discovery of mutants in *Arabidopsis* that are considered to be defective in the biosynthesis of brassinolide (Ecker, 1997). These plants show in darkness a morphology that is very close to that of light-grown plants; for that reason defects in genes encoding signal substances involved in light regulation of growth was suggested. Treatment with Bl of these mutants restored the normal etiolated phenotypes grown in the dark and also initiated their normal wild-type growth in the light. When the genes responsible for this type of development

were cloned, it was found that one of them, characteristic for the *deetiolated 2 (det 2)* mutant, encoded a protein that was similar to the mammalian enzyme that catalyzes the 5α-reduction step of steroid biosynthesis. Another one, related to the *constitutive photomorphogenesis and dwarf (cpd)* mutant, was found to encode a protein with similarity to cytochrome P450/steroid hydroxylase, responsible for one of the later steps in the biosynthesis of steroids. It was experimentally confirmed that both the *det 2* and the *cpd* mutants were defective in steroid biosynthesis but in different steps. The fact that exogenous Bl was able to restore the wild-type phenotype was interpreted as a confirmation of the deficiency of Bl in these mutants. These data are very important as new evidence for the role of BS in plant development.

G. MECHANISM OF ACTION, RECEPTION, AND TRANSPORT

The mechanism of action has been an attractive target for researchers since the elucidation of the structure of Bl, but for many reasons only recently has some significant progress in this direction been achieved. Although this problem is still rather far from its final solution, many important data have brought us closer to understanding the mode of regulatory action of BS.

Taking into account the high variability of the physiological effects of BS, it is probable that more than one molecular mechanism of their action exists. Two main aspects of the primary mechanism have to be considered first: an effect of BS on the biosynthesis of enzymes *via* an effect on genome expression and an effect of BS on membranes. The first effect is responsible for the slow reactions of plants on exogenous hormones, and the second one for the quick reactions. The data discussed in the previous sections of this chapter showed that the complicated character of BS-initiated processes is probably caused by both types of action with overlap and close interconnections between them.

The fact that not long before the discovery of Bl the main postulates of steroid hormone action in mammalians were formulated (Roy and Klark, 1980) was important for researchers who were interested in mechanisms of hormone action. Since the idea of hormone regulation in plants came from human endocrinology, these mechanisms were considered to be applicable also for plant hormones (Muromtsev *et al.*, 1987), and in particular for BS (Kalinich *et al.*, 1985, 1986). This point of view was purely hypothetical but recently, after obtaining evidence for BS-induced gene expression, this traditional mechanism of steroid hormone action in application to BS became more realistic.

The molecular genetic approach has played an important role in bringing the insight in the mechanism of action of BS to a higher level. While the first investigations in this direction led to the recognition of gene expression under the action of BS (Kulaeva *et al.*, 1989, 1991; Clouse and Zurek, 1991), further developments have resulted in the identification of the BS up-regulated gene (BRU1) from soybean. It was found that the expression of BRU1 was specifically initiated by BS that were structurally close to Bl, but not by other plant hormones or steroids (Clouse *et al.*, 1992; Zurek *et al.*, 1994). The significant sequence homology of BRU1 to xyloglucan endotransglycosylase (XET), an enzyme involved in the regulation of some cell wall components, indicated a possible role of BRU1 in cell expansion that is realized *via* an increase of the activity of XET and loosening of the cell wall (Clouse, 1996a).

If gene expression under BS action in plants is similar to the steroid-regulated gene expression in animals and a chain of events such as

BS + receptor → BS-receptor complex → nuclear DNA → mRNA → protein (enzyme)

exists, a detection of the Bl receptor is an actual challenge. The history of classical phytohormone receptor studies, which is much longer than the history of BS, does not allow us to suppose that a solution for this problem can be found easily, because even for these much better investigated hormones the traditional

approach based on hormone-binding experiments led to the structure elucidation of only one auxin receptor.

Substantial progress in this direction has been achieved by application of a new methodology, developed after the isolation of *Arabidopsis* hormone-response mutants (Ecker, 1997). Some of these mutants grown in the dark or in the light, with phenotypes similar to those observed in hormone-deficient mutants, showed a nearly normal response to the usual phytohormones but were found to be insensitive to Bl (Clouse and Langford, 1995; Clouse *et al.*, 1996a; Kauschmann *et al.*, 1996a,b; Li and Chory, 1997). Study of the mutants led to the identification of only one Bl signaling gene that was assumed to be encoding a protein similar to the steroid receptor of animals. The cloning of this gene, called BRI1, and further elucidation of the protein structure showed its similarity to known receptor-like molecules, but not to those that were known previously to participate in steroid signaling events (Li and Chory, 1997). BRI1 is a member of a family of receptor-like transmembrane kinases that have structural similarity with a variety of proteins that are widespread from bacteria to human and are involved in protein-protein interactions. Although their interactions with nonprotein ligands are not yet known, some specific structural features of BRI1 suggest it has a role either in the direct binding of Bl or in binding of a complex of Bl with an intermediary protein. Intensive ongoing research to solve this and related problems seems very promising to get a confirmation of BRI receptive function in the very near future. In this way its real mode of action, membrane localization, and the targets to which its intracellular signals are transmitted can be elucidated.

To answer the questions about the action of endogenous BS in plants, knowledge about their transport and localization of biosynthesis is necessary. Unfortunately, there are very few data about these subjects in the literature now. Very useful for the clarification of the last question could be the application of highly sensitive analytical approaches based on immunochemical methods to

study the subcellular localization of BS. Although such approaches are widely used for the microanalysis of several kinds of antigens as well as for phytohormones and a first attempt of their application to BS was done rather long ago (Horgen *et al.*, 1984), this methodology became accessible for the BS series just recently. It became possible after solving the problems of chemical synthesis of BS, their haptens, and conjugates and preparation of antibrassinosteroid antibodies (Yokota *et al.*, 1990b; Schlagnhaufer *et al.*, 1988, 1991; Naren *et al.*, 1996).

The approach using polyclonal antibodies against castasterone to study the localization of BS in germinating pollen of *Brassica napus* showed a specific binding of BS to nuclear components (Smith *et al.*, 1992). The detection of strong labeling in the amyloplasts suggested a role of plastids as storage organelles for BS (Sasse *et al.*, 1992). This supposition was further supported by the study of pollen of ryegrass (*Lolium perenne*) in different stages of development. It was found that BS were increasingly accumulated in starch granules during amyloplast maturation and this can be considered as an indication for the storage function of these organelles for BS (Taylor *et al.*, 1993). The detection of heavy labeling within starch granules and in the zone closely connected to it in the bicellular stage of pollen development, when the differentiation of proplastids is not yet finished and the stromal tissue is partially maintained in the starch granules, led to the conclusion that BS might be synthesized in the stroma, whose location close to the starch granule would allow the absorption of BS in these particles. An easy release of BS from starch granules was shown to be possible after hydration and that could make these compounds available to affect the process of pollen germination.

In the case of *Brassica napus* and *Lolium perenne* no specific binding of BS to any soluble proteins from pollen extract was found (Smith *et al.*, 1992; Taylor *et al.*, 1993). This might be interpreted as the absence in the cytoplasm of a specific cytoplasmic receptor as suggested earlier as a means of BS

transportation to the nucleus (Kalinich *et al.*, 1985, 1986). In this connection, the preparation of antibodies to Bl and a comparative study of different types of conjugates based on differently modified Bl as possible antigens in anti-BS antibody induction will be important tasks for the future because it will increase the sensitivity and selectivity of the analysis.

A short-distance auto- or a paracrine-like way of interaction, in terms of animal endocrinology (Muromtsev and Danilina, 1996), between BS and their targets is probable also for the effects of endogenous hormones in the plant reproductive system and for exogenously applied BS. Endocrine interaction, which assumes long-distance transportation of the hormones *via* the xylem, has not yet been found to be important for the realization of the endogenous effects of BS. Nevertheless, such a long-distance transport probably *via* the xylem of exogenously supplied labeled BS that were translocated from the roots to the shoots was shown to take place in rice (Yokota *et al.*, 1992) and in cucumber and wheat (Nishikawa *et al.*, 1994). When labeled Bl and Bk were applied to the leaf surface of rice, only a slow transport of these compounds or their metabolites from the leaves to the roots was observed (Yokota *et al.*, 1992).

The question about BS transport proteins that might be similar to the corresponding ones of mammalian hormones is still open. The existence of special transport forms of BS similar to those in gibberellins is still uncertain. Data (Yokota *et al.*, 1992) on different rates of transportation obtained for natural BS with various structures of the cyclic part might be considered as an indication of such a possibility.

New possibilities for study of the mechanism of the action of BS became accessible due to the discovery of the first selective inhibitor of BS, which was found recently in the fungus *Drechslera avenae* (Kim, S.-K. *et al.*, 1994a, 1995).

H. OTHER EFFECTS OF BS

As mentioned in Chapter II, BS are very close structurally to ecdysteroids (ES), which are the moulting hormones (MH) of insects and other arthropods. ES are widespread in both the animal and plant kingdoms. This structural similarity and the corresponding possibility to bind ES receptor were the reasons which initiated the search for MH-like or anti-MH-like properties in BS series.

The first study of MH activity of BS was done using the test with imaginal disks isolated from the fly *Phormia terra novae* (Hetru *et al.*, 1986). Some BS showed a promoting effect on the evagination of imaginal discs but at concentrations 10-100 times higher than those of ecdysterone. An inhibition of ecdysterone action by BS was observed, and this agonistic effect of Bk and (22*S*,23*S*)-HBl was maximal at a concentration of $5 \cdot 10^{-5}$ M when the action of ecdysterone was fully inhibited. This fact indicated possible competition between BS and ES for the hormone receptor. It was confirmed in experiments on the competitive binding of two BS analogs [(22*S*,23*S*)-HBl and (22*S*,23*S*)-HBk] and radiolabeled ponasterone A to the ES receptor obtained from the blowfly *Calliphora vicina* larvae (Lehmann *et al.*, 1988). Ponasterone A is an ES ligand which is able to bind to the ES receptor with a high affinity (K_D 10^{-9} M). The affinity of (22*S*,23*S*)-HBk showed a K_D of about $5 \cdot 10^{-6}$ M and the binding of BS was reversible.

The antiecdysteroid activity was found also when BS analogs were applied to the cockroach *Periplaneta americana* (Richter *et al.*, 1987). It was shown that after feeding on a special diet containing (22*S*,23*S*)-HBl, a delay of 11 days took place in the moult of final-instar nymphs. (22*S*,23*S*)-HBk did not exhibit such action, but the extract of rape flowers, a natural source of brassinolide, also delayed the moult by 9 days. The finding of similarity in the action of natural extract and artificial BS analog was important in confirming the idea that the steric orientation of the hydroxy functions in the side chain of BS has no

significant influence on their binding to the ES receptor, and both natural and synthetic hormones interact with the receptor in a similar way. However, this fact alone should not be overestimated because a significant amount of other steroid substances was contained in the extract, and the confirmation could be even more reliable if Bl or HBl was compared with (22*S*,23*S*)-HBl directly.

The fact that BS and their analogs possess anti-MH activity in insects stimulated a desire to learn whether they have other typical for ES types of activity, such as a neurotropic effect. This was investigated on isolated brains of last-instar larvae of the cockroach *Periplaneta americana* for (22*S*,23*S*)-HBl and (22*S*,23*S*)-HBk under *in vitro* conditions (Richter and Adam, 1991). The effect of both BS analogs was found to be similar to the effect of 20-OH-ecdysone. However, to reach the same level of response, a three fold higher concentration of (22*S*,23*S*)-HBk and a tenfold higher concentration of (22*S*,23*S*)-HBl were necessary. These results could be considered as an indication of the affinity of BS to neuronal ecdysteroid binding sites, which are the targets for 20-OH-ecdysone in insect brains. This means that BS possess ES agonist-like activity and their MH-inhibiting effect is realized probably not only at epidermal ES binding sites but also in the central nervous system. Similar results on the competition of BS with ES for the intracellular ES receptor from the epithelial cell line from *Chironomus tentans* were obtained with the same compounds (Spindler *et al.*, 1991), but they showed no pronounced ES-agonistic or ES-antagonistic properties in other model systems (Charrois *et al.*, 1996). A study of BS action on the viability and microfilarial production of adult *Brugia pahangi* cultured *in vitro* showed a behavior that was different from other studied compounds which apparently disrupted hormonally regulated processes in insects (Barker *et al.*, 1989). Similar to these compounds BS inhibited microfilarial production but did not exhibit filaricidal activity and did not influence the worm viability.

The analysis of BS effects in insects led to the conclusion that they are the first true antiecdysteroids found, and their properties and natural origin were considered to be very important for potential application in insect pest control as new safe substances, which represent the "third-generation pesticides" (Richter and Koolman, 1991).

Until recently, there were practically no attempts to investigate BS action in vertebrates except studies on the toxicology of BS. The obtained results reflected the very low toxicity of BS and their inactivity in tests for other negative influences on animals. Possible stimulating properties of BS similar to those known in the ecdysteroid series became the subject of interest only recently. The first study in this direction showed a pronounced toxicoprotective effect of EBl on Russian sturgeon *Acipenser gueldenstaedti* fingerlings (Vitvitskaya *et al.*, 1997a). Treatment of fingerlings with EBl solution at a concentration of 10^{-4} mg/l for 2 h before their 48-h exposure to solutions of toxicants such as $CuSO_4$ (0.004 mg/l), phenol (0.1 mg/l) and the detergent "Lotos" (0.2 mg/l) showed a significant decrease of the negative influence of the toxicants on the fingerlings in regard to swiming activity and ability to resist a current, reactivity to a sonic signal, and training. The effect of EBl was higher than the effect of known protectants, which were compared with EBl in the same experiments.

Since unfavorable factors such as pollutants, oxygen deficiency, high salinity, and others influence negatively the development and propagation of sturgeons, the revealed protective properties of EBl were investigated further. It was found (Vitvitskaya *et al.*, 1997b) that the treatment of Russian sturgeon, white (Pacific) sturgeon *A. transmontanus*, and starred sturgeon *A. stellatus* eggs with EBl at a concentration of 10^{-5}-10^{-9} mg/l significantly increased fecundation, hatching, and larvae survival (Fig. 61).

The data summarized in Fig. 61 show that the effect of EBl on the eggs was higher in unfavorable conditions and especially in the case of the starred sturgeon. The fingerlings grown from EBl-treated eggs had better morphological

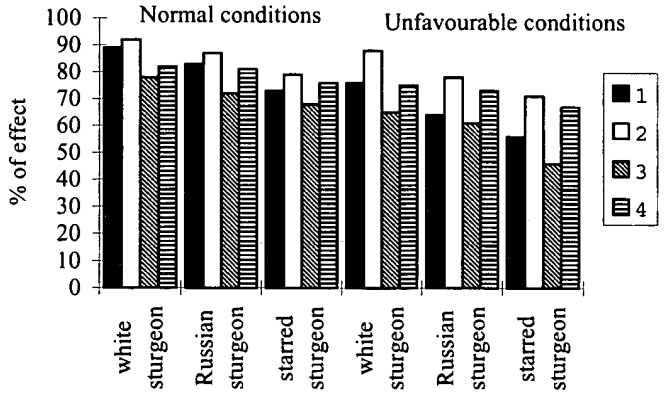

Fig. 61. Effect of EBl on sturgeon egg fecundation and hatching (1 - fecundation, control; 2 - fecundation, EBl; 3 - hatching, control; 4 - hatching, EBl).

characteristics and better resistance to stress factors, particularly to salt stress conditions.

The treatment of the sturgeon fish larvae with EBl increases the survival of the fingerlings. This effect for some species of sturgeons is illustrated by Fig. 62. Along with better survival of fingerlings, a tendency to increase of body weight was observed, and it was the most significant in the case of starred sturgeons, which were cultivated under worse hydrochemical and temperature conditions in

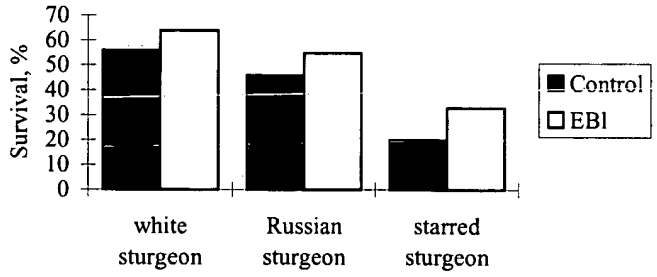

Fig. 62. Effect of EBl on the survival of sturgeon fingerlings.

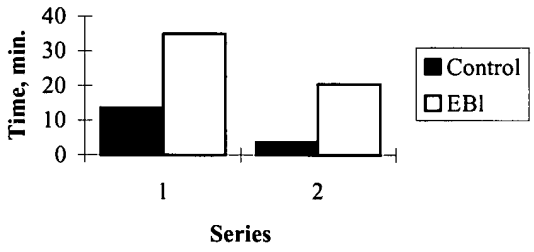

Fig. 63. Effect of EBl on spermatozoon viability time of unconserved (1) and cryoconserved (2) sperm in the Russian sturgeon.

comparison with other fishes.

The discovery of the influence of BS on the reproductive physiology of sturgeons stimulated investigations in this field, which showed prospects of wider employment of BS in aqua cultures. Thus, it was found that the application of EBl enhances efficiently the activity and viability of spermatozoons, which is very important in animal breeding. An example of such application is the activation of spermatozoons of the Russian sturgeon in normal conditions and after cryoconservation. Figure 63 illustrates the effect of EBl on the sperm from the males of Russian sturgeon (Vitvitskaya *et al.*, 1997c). The activity and viability of spermatozoons in the samples of unconserved sperm treated with EBl at a concentration of 10^{-6} mg/l were about 250% of control. The treatment of cryoconserved sperm gave a corresponding value of about 550% of control.

The data on the action of EBl on fish physiology seem very valuable for clarifying the possible responses to BS in vertebrates. Although being the first clue, these data promise further findings that may become important for humans, if the spectrum of stimulative action of BS in vertebrates is as broad as in plants.